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Award Number: DAMD17-00-1-0483

TITLE: Regulation of ErbB-2 Metabolic Stability

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REPORT DATE: March 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2004	Final (1 Mar 2000 - 28 Feb 2004)

4. TITLE AND SUBTITLE
Regulation of ErbB-2 Metabolic Stability

5. FUNDING NUMBERS
DAMD17-00-1-0483

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

This grant has focused on the mechanism by which geldanamycin controls the metabolic stability of ErbB-2, a co-receptor tyrosine kinase. ErbB-2 is overexpressed in breast cancer and is known to increase cell proliferation. Geldanamycin decreases ErbB-2 levels in cells and is in clinical trials for the treatment of breast cancer.

Our studies in this grant have examined the mechanism by which geldanamycin decreases ErbB-2 levels. Published data demonstrate that geldanamycin provokes proteolytic cleavage events within the ErbB-2 intracellular domain and thereby decreases the level of the mature form of ErbB-2. One of these cleavage events occurs in the carboxyterminal domain of ErbB-2, while another takes place in the kinase domain. We have identified sequences within the kinase domain that are required for this molecule's sensitivity to geldanamycin. Once these initial cleavage events occur the resultant fragments of ErbB-2 are rapidly degraded. However, these fragments may induce novel biologic activities within cells particularly if ErbB-2 is overexpressed. We have identified a short sequence within the kinase domain of ErbB-2 that is able to induce cell death. This could account for the cellular toxicity of geldanamycin.

14. SUBJECT TERMS
Cancer biology, receptor tyronsine kinase, protein degradation

15. NUMBER OF PAGES
43

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

ErbB-2 is a receptor tyrosine kinase that by heterodimerization mediates signal transduction by the EGF and heregulin growth factors and then receptors ErbB-1, ErbB-3, ErbB-4 (1). Both ErbB-1 and ErbB-2 are functionally important in breast cancer and are being pursued as clinical therapeutic targets (2). Geldanamycin is being tried in clinical trials because it enhances the loss of ErbB-2 from cells and thereby decreases their growth capacity (3). The focus of this study has been to elucidate the mechanism by which geldanamycin acts on ErbB-2 so as to decrease its level in cells.

BODY

Following submission of our application to seek funding for this project in May 1999, we continued to explore the issue of ErbB-2 metabolic destabilization by geldanamycin. These efforts resulted in a publication (4) that was submitted in April, 2000, just one month after the initiation of support from the Department of Defense. Since the experiments in that paper were all completed prior to the initiation of DOD support, that publication does not show DOD support. However, the results do bear on the specific aims proposed and, hence, the main points of the manuscript are cited below.

The results of this manuscript (4) are directly related to our initial Statement of Work: Task 1, Task 2A and Task 2C. In this regard we show that geldanamycin-induced ErbB degradation involves the entire cellular pool of ErbB-2. More importantly, this manuscript shows that geldanamycin-induces the endoproteolytic fragmentation of ErbB-2 into at least two stable fragments. One fragment of 135 kDa represents the ErbB-2 ectodomain, the transmembrane domain and a small portion of the cytoplasmic domain. The second fragment of 23 kDa represents the carboxyterminal region of the ErbB-2 cytoplasmic domain. These data also showed that these two fragments are subsequently degraded by two different intracellular proteolytic systems. The 135 kDa membrane-localized fragment is degraded by the lysosome, while the smaller cytosolic 23 kDa fragment is degraded by the proteosome. These data show, therefore, that geldanamycin-induced degradation of ErbB-2 involves at least two major proteolytic systems.

The data in this manuscript also show the following relevant points. That the kinase domain of ErbB-2 is required for geldanamycin-induced degradation was demonstrated by the analysis of chimeric molecules and deletion mutants. That the carboxyterminus of ErbB-2 exerts a protective influence on ErbB-2 sensitivity to geldanamycin was shown by the evaluation of cytoplasmic domain fragments of ErbB-2 expressed in cells as GFP-fusion proteins.

A second manuscript (5), credited entirely to DOD support, addressed the issues identified in Task 2B. The work in this paper began by determining the N-terminal sequence of the geldanamycin-induced 23 kDa fragment derived from the ErbB-2 carboxyterminal region. The sequence indicated that the amino terminus of this fragment corresponded to Gly1126 in ErbB-2. Analysis of the residues immediately preceding Gly1126 suggested the presence of a caspase cleavage site and site-directed mutagenesis of Asp1125 prevented geldanamycin-induced formation of the ErbB-2 23 kDa fragment in cells treated with geldanamycin. However, when formation of the 23 kDa fragment was blocked by mutagenesis or caspase inhibition, ErbB-2

degradation in the presence of geldanamycin still took place. This cleavage, therefore, is not essential for destabilization of ErbB-2 by geldanamycin. That caspase activities take part in geldanamycin-induced ErbB-2 degradation was somewhat surprising. Caspases are intimately associated with programmed cell death and geldanamycin-induced apoptosis is not widely reported, though there is evidence that it can occur in some cells treated with this drug.

Subsequently, we assayed various compounds that are known to be inducers of apoptosis, particularly in mammary carcinoma cells. Some of these, such as paclitaxel, had no effect on ErbB-2 metabolic stability. However, both staurosporin and curcumin did induce ErbB-2 degradation with kinetics similar to geldanamycin and with formation of the same 23 kDa carboxyterminal ErbB-2 fragment. Interestingly, when formation of the 23 kDa fragment was blocked by addition of the pan-caspase inhibitor, the staurosporin-induced degradation of ErbB-2 was prevented. Therefore, caspase activity is essential to the staurosporin-induced degradation of ErbB-2, but not, as previously mentioned, to geldanamycin-induced ErbB-2 degradation.

During the past year our efforts have concentrated on the cleavage that produces the 135 kDa fragment of ErbB-2. As previously described (4), this fragment includes the ectodomain, transmembrane domain, and part of the kinase domain. Our previous work (4) and that of others (6) also concluded that the kinase domain of ErbB-2 is necessary for geldanamycin-induced degradation of this co-receptor molecule. Since the ErbB-1 molecule, a close relative of ErbB-2, is not sensitive to geldanamycin-induced degradation, we compared sequence homologies of the kinase domains of ErbB-1 and ErbB-2. Based on that analysis residues unique to ErbB-2 were chosen for mutagenesis. The data published in a DOD-supported manuscript (6) showed that if three residues (Leu784, Leu785, Ile788) are mutated to alanine, the resultant ErbB-2 mutant protein is not sensitive to geldanamycin-induced degradation. We also identified a second nearby motif that confers geldanamycin-sensitivity in ErbB-2. This is the sequence between Gly776 and Ser783 of ErbB-2. Multiple residues in this motif were mutagenized to correspond to the analogous residues in ErbB-1 produces an ErbB-2 mutant that is no longer sensitive to geldanamycin. These combined results show that a specific region (residues 776-788) within the ErbB-2 kinase domain are required for geldanamycin-induced degradation.

To further test these results we made several C-terminal deletion mutations in ErbB-2. When these constructs were expressed, we found that loss of residues beyond residue 783 dramatically destabilizes ErbB-2, indicating kinase domain sequences not only mediate geldanamycin-induced degradation of ErbB-2, but also control the basal level of ErbB-2 metabolic stability in cells.

Drugs that induce the fragmentation of ErbB-2 (such as geldanamycin, staurosporin, curcumin) frequently induce apoptosis. We have also found that antibodies to the ErbB-2 ectodomain induce both cell death and fragmentation in or near the kinase domain. Likewise, the addition of EGF to a chimeric receptor having an ErbB-1 ectodomain and an ErbB-2 cytoplasmic domain induces fragmentation and cell death. We hypothesized that perhaps the ErbB-2 kinase domain contains cryptic sequences that, if exposed following fragmentation of the receptor, would induce cell death. To test this hypothesis we constructed fusion proteins between green fluorescent protein and segments of ErbB-2. The results are presented in detail in reference 7. The fusion proteins were then expressed in COS7 cells and CHO cells. Constructs containing sequences derived from the N-terminal lobe of the ErbB-2 kinase domain were observed to

induce cell death with characteristics similar to apoptosis. More specifically when residues 766-802 of the ErbB-2 kinase domain are expressed with as a GFP fusion protein, nearly all cells die within 2-3 days. If just GFP is expressed, there is no cell death. These results, therefore, indicate that sequences within the ErbB-2 kinase can provoke cell death, if ErbB-2 is fragmented so as to expose these sequences. Interestingly, analogous sequences from several other receptor tyrosine kinases (ErbB-1, ERbB-4, TrkA, VEGFR1) also induced cell death. These results imply that geldanamycin may induce cell death by liberating these sequences from ErbB-2 in the process of provoking proteolytic cleavage reaction described above. The research described above is directly related to Tasks 2A, B, and C in the original proposal.

Finally, we have tested cell death initiated by geldanamycin and a derivative of it known as 17-AAG, which is reported to be less toxic for cells (3). The problem in clinical trials of geldanamycin has been its toxicity. We evaluated the concentrations of geldanamycin and 17-AAG that provoke cell death in MCF-7 cells, which have a low level of ErbB-2, and MCF-7 cells transfected to express a high level of ErbB-2. We found that both compounds were equally toxic to both cell lines. Hence, toxicity to cells could not be separated on the basis of the cellular expression of ErbB-2. It seems likely, therefore, that the toxicity of geldanamycin and 17-AAG are not related to the cellular level of ErbB-2, but to some other action of this compound.

KEY RESEARCH ACCOMPLISHMENTS

- The kinase domain of ErbB-2 is required for ErbB-2 degradation induced by geldanamycin
- The carboxyterminus of ErbB-2 has a protective influence on ErbB-2 stability in the presence of geldanamycin
- Geldanamycin-induces the degradation of the entire cellular population of ErbB-2
- The initial step in geldanamycin-induced degradation is endoproteolytic fragmentation
- ErbB-2 fragments of 135 kDa (membrane-bound) and 23 kDa cytosolic are formed in the presence of geldanamycin
- Formation of the 23 kDa ErbB-2 fragment in the presence of geldanamycin requires caspase activity
- Some inducers of apoptosis, such as staurosporin, also induce ErbB-2 degradation and formation of the 23 kDa fragment
- Caspase inhibition prevents staurosporin, but not geldanamycin-induced ErbB-2 degradation
- Sequences within residues 776-788 of the ErbB-2 tyrosine kinase domain are necessary for geldanamycin-induced degradation

- ErbB-2 kinase domain mutants that are resistant to geldanamycin-induced degradation have been constructed
- Loss of residues beyond residue 783 in the ErbB-2 kinase domain results in a fragment that is metabolically unstable in cells
- A small sequence of 38 residues from the N-terminal region ErbB-2 kinase domain provoke cell death when expressed in cells

REPORTABLE OUTCOMES

Manuscripts:

1. Tikhomirov, O., and Carpenter, G. (2000). Geldanamycin Induces ErbB-2 Degradation by Proteolytic Fragmentation. *J. Biol. Chem.* 275: 26625-26631. (not credited to DOD funding)
2. Tikhomirov, O. and Carpenter, G. (2001). Caspase-dependent Cleavage of ErbB-2 by Geldanamycin and Staurosporin. *J. Biol. Chem.* 276: 33675-33680.
3. Tikhomirov, O., and Carpenter, G. (2003). Identification of ErbB-2 Kinase Domain Motifs Required for Geldanamycin-Induced Cell Death. *Cancer Res.* 63: 39-43.
4. Tikhomirov, O. and Carpenter, G. (2004). Identification of Proteolytic Fragments from ErbB-2 that Induce Apoptosis (submitted).

Presentations:

1. Tikhomirov, O. and Carpenter, G. (2000). "Geldanamycin Induces Degradation in Lysosomes." *FASEB J.* 14: A1544. Poster presentation at American Society for Biochemistry and Molecular Biology Annual Meeting, Boston, MA.
2. Tikhomirov, O. and Carpenter, G. (2002). Proteolytic Fragmentation of ErbB-2. Poster Presentation at Era of Hope Department of Defense Breast Cancer Research Program Meeting, Orlando, FL.

CONCLUSIONS

Geldanamycin induces a decrease in cellular levels of ErbB-2 by provoking a series of proteolytic cleavage events within the intracellular domain of ErbB-2. One cleavage occurs near the carboxyterminus and is executed by caspases. The other major cleavage seems to occur within the kinase domain. Kinase domain mutations can be made in ErbB-2 that prevent any cleavage events provoked by geldanamycin. This indicates that a geldanamycin binding protein, such as hsp90, may interact with these kinase domain sequences and, in the absence of geldanamycin, prevent ErbB-2 degradation.

The ErbB-2 fragments produced in the presence of geldanamycin are degraded by cellular proteolytic systems, which may produce additional fragment of smaller size. Sequences within the 135 kDa fragment of ErbB-2 are highly toxic to cells and could be liberated during this proteolytic processing. This could account, at least in part, for the cellular toxicity of geldanamycin.

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5. Tikhomirov, O. and Carpenter, G. (2001). Caspase-dependent Cleavage of ErbB-2 by Geldanamycin and Staurosporin. *J. Biol. Chem.* 276: 33675-33680.
6. Tikhomirov, O. and Carpenter, G. (2003). Identification of the ErbB-2 Kinase Domain Motifs Required for Geldanamycin-induced Degradation. *Cancer Res.* 63: 39-43.
7. Tikhomirov, O. and Carpenter, G. (2004). Identification of Proteolytic Fragments from ErbB-2 that Induce Apoptosis. (submitted).

APPENDICES

Attached are three reprints and one preprint of manuscripts.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 34, Issue of August 25, pp. 26625–26631, 2000
Printed in U.S.A.

Geldanamycin Induces ErbB-2 Degradation by Proteolytic Fragmentation*

Received for publication, April 11, 2000, and in revised form, May 25, 2000
Published, JBC Papers in Press, June 20, 2000, DOI 10.1074/jbc.M003114200

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Exposure of carcinoma cell lines to the antibiotic geldanamycin induces the degradation of ErbB-2, a co-receptor tyrosine kinase that is frequently overexpressed in certain tumors. Using ErbB-2 mutants expressed as chimeric receptors or green fluorescent protein fusion proteins, we report that the kinase domain of ErbB-2 is essential for geldanamycin-induced degradation. The kinase domain of the related epidermal growth factor receptor was not sensitive to this drug. The data further indicate mechanistic aspects of ErbB-2 degradation by geldanamycin. The data show that exposure to the drug induces at least one cleavage within the cytoplasmic domain of ErbB-2 producing a 135-kDa fragment and a 23-kDa fragment. The latter represents the carboxyl-terminal domain of ErbB-2, whereas the former represents the ectodomain and part of the cytoplasmic domain. Degradation of the carboxyl-terminal fragment is prevented by proteasome inhibitors, whereas degradation of the membrane-anchored 135-kDa ErbB-2 fragment is blocked by inhibitors of the endocytosis-dependent degradation pathway. Confocal microscopy studies confirm a geldanamycin-induced localization of ErbB-2 on intracellular vesicles.

ErbB-2 is a Type 1 transmembrane tyrosine kinase that functions as a co-receptor by forming dimers with other members of the ErbB receptor family (ErbB-1 (EGF[1] receptor), ErbB-3, and ErbB-4; Refs. 1 and 2). Although ErbB-2 has a potential ligand-binding ectodomain, no direct ligand has yet been identified. In its role as a co-receptor, ErbB-2 enhances the signaling capacity of its dimerization partners. The association of ErbB-2 with these various receptors is, however, entirely ligand-dependent. In the absence of growth factor ErbB-2 is reported to interact with CD44, an adhesion receptor, in ovarian carcinoma cell lines (3) and with a large plasma membrane glycoprotein complex in microvilli of a mammary adenocarcinoma cell line (4). ErbB-2 has also been demonstrated to form ligand-dependent complexes with the IL-6 receptor component gp130 (5) and Trk A (6), the nerve growth factor receptor.

ErbB-2 was originally identified as the transforming oncogene *neu* in which a point mutation in the transmembrane domain is responsible for its oncogenic potential (7, 8). ErbB-2 also functions as an oncogene when overexpressed (9, 10) and in humans is frequently overexpressed in breast and ovarian tumors (11). ErbB-2 overexpression in breast cancer is associated with a poor prognosis (12), and hence it is a target for therapeutic reagents, including monoclonal antibodies and drugs (13). Frequently, antibodies that decrease the growth of ErbB-2-expressing tumors also reduce the level of ErbB-2 by a mechanism that is unclear. Hence, the transforming activity of ErbB-2 is related to structural changes or changes in its level of expression.

The benzoquinoid ansamycin antibiotics geldanamycin and herbimycin were first isolated from the culture broths of several actinomycete species (14, 15) and described as inhibitors of tyrosine kinase-dependent growth (16, 17). These compounds, particularly geldanamycin, have tumorical activity toward numerous tumor cell lines (18), including those that overexpress ErbB-2 (19). This action toward tumor cell lines is attributed to the capacity of geldanamycin to induce the degradation of several important signal transducers important in mitogenic pathways. These targets include protein kinases, such as Src, Raf, FAK, and ErbB-2, and other growth regulating proteins, such as p53 (20). The mechanism for the geldanamycin-induced degradation of these various molecules is centered on the Hsp90 family of chaperones, because Hsp90 is the major intracellular protein that binds geldanamycin (20, 21). Geldanamycin has been shown to dissociate Hsp90 from various proteins and thereby inhibit their function, such as the nuclear translocation of glucocorticoid receptors, or to induce their metabolic degradation, such as Src, Raf, and p53.

In the case of ErbB-2, association with Hsp90 has not been reported. However, it has been reported that the glucose-regulated chaperone GRP94, an Hsp90 family member that is localized to the lumen of the endoplasmic reticulum, does associate with ErbB-2 in a geldanamycin-sensitive manner (22). Geldanamycin-induced degradation of ErbB-2 is reported to involve, presumably as a consequence of the dissociation of GRP94, the polyubiquitination of ErbB-2 and its proteosomal degradation (23). On the basis of GRP94 localization, these studies would suggest an interaction with the ErbB-2 ectodomain in the lumen of the endoplasmic reticulum and drug-induced degradation during receptor biosynthesis. Others, however, have suggested that this interpretation does not account for the quantitative aspects of ErbB-2 degradation induced by geldanamycin (24).

We have explored the question of how geldanamycin induces ErbB-2 degradation and show that the ErbB-2 kinase domain is essential for sensitivity of geldanamycin. Also we show that geldanamycin induces fragmentation of ErbB-2 within the carboxyl-terminal region of the cytoplasmic domain and that the

* This work was supported by National Institutes of Health Grant CA24071. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; ALLN, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal; GFP, green fluorescent protein; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

resulting transmembrane fragment is degraded by a mechanisms that involves the formation of intracellular vesicles.

EXPERIMENTAL PROCEDURES

Materials—Geldanamycin, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN), folimycin, proteasome inhibitor I, and 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulfone were purchased from Calbiochem. Batimastat (BB94) was a generous gift of Dr. P. Dempsey (Vanderbilt University, Nashville, TN). TAPI-O was obtained from Peptide International. Other protease inhibitors, protein G or protein A, and ECL reagents were from Sigma. Monoclonal antibody (Ab5) against ErbB-2 extracellular domain and monoclonal antibody (Ab3) against carboxyl terminus of ErbB-2 were purchased from Oncogene Science. Monoclonal antibodies against EGFR extracellular domain (Ab13 and Ab14), monoclonal antibody (Ab2) against the ErbB-4, and monoclonal antibody (Ab8) against ErbB-2 cytoplasmic domain were from NeoMarkers. Goat anti-mouse IgG labeled with Alexa 488 for confocal microscopy was from Molecular Probes. Human ErbB-2 cDNA and NIH 3T3 cell lines with overexpressed ErbB-2 or EGF receptor/ErbB-2 chimeric receptors were generous gifts from Dr. Pier Paolo Di Fiore (European Institute of Oncology, Milan, Italy) and have been previously described elsewhere (25). The mammalian expression vector pEGFP-C1 for heterologous fusion proteins to green fluorescent protein (GFP) and monoclonal antibody to GFP were obtained from CLONTECH.

Cell Culture and Transfection—Human mammary tumor-derived SKBr3 cells were grown in 5% CO_2 at 37 °C in McCoy medium with 10% fetal bovine serum, COS 7 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, and all fibroblast cell lines were grown in Dulbecco's modified Eagle's medium containing 10% calf serum. Cells were grown to about 80% confluency then washed two times, and treated with indicated drugs in serum-free Dulbecco's modified Eagle's medium. For transfection and expression of GFP fusion protein, COS 7 cells were grown to ~70% confluency overnight and transfected with LipofectAMINE (Life Technologies, Inc.) according to manufacture's recommendations (10 μg of plasmid DNA mixed with 16 μl of LipofectAMINE were used per 60-mm tissue culture dish). The cells were grown for 48 h before assays.

Construction of GFP Fusion Proteins—The ErbB-2 kinase domain and cytoplasmic region cDNA fragments were generated by PCR with high fidelity VENT^R DNA polymerase (New England BioLabs). Following the numbering of Yamamoto *et al.* (26), the kinase domain fragment and cytoplasmic domain fragment correspond, respectively, to residues 715–990 and 676–1255 of ErbB-2. To prepare these two fragments the following primers were synthesized: upstream primer with *Sac*I restriction site 5′-GGG ATC CTC ATC AAA CGA GCT CAG AAG ATC-3′ (primer 1), downstream primers with *Xba*I restriction sites 5′-ACT ACG TCC AGT TCT AGA TCA CAC TGG CAC GTC CAG ACC-3′ (primer 2), 5′-GTA GAA GGT GCT GTC TAG AGG ACT GGC TGG-3′ (primer 3). PCR products were cloned into pEGFP-C1 vector by using *Sac*I and *Xba*I sites.

Constructs with ErbB-2 carboxyl terminus and its truncation fragments fused to GFP were prepared by megaprimer approach. The carboxyl-terminal domain fragment corresponds to residues 991–1255 or ErbB-2 (26). In the first round of PCR the following primers that contain stop codons were used as 5′ primers: 5′-GTA CCC CTG CCC TAA GAG ACT GAT GGG-3′, 5′-CAG CCC CCT TAA CCC CGA GAG GGC-3′, 5′-CCC CAG TAC TAA ACA CCC CAG GGA-3′, 5′-CCC AGC ACC TAA AAA GGG ACA CCT-3′ and primer 2 as the 3′ primer. One strand of this product was used as the 3′ primer in a second round of PCR with primer 1.

The following primers were used to prepare construct of GFP fusion protein with EGF receptor kinase domain (residues 683–698, according to the numbering of Ullrich *et al.* (27)): 5′-CGA AGG CGC CAC AGA GCT CGG AAG CGC ACG-3′ (upstream primer with *Sac*I restriction site) and 5′-GTA GAA GTT GGA GTC TAG AGG ACT TGG-3′ (downstream primer with *Xba*I restriction site). The cDNA of ErbB-2 carboxyl terminus (residues 991–1255, according to Yamamoto *et al.* (26)) was prepared with 5′-TCC CGC ATG GCC AGA GCT CCC CAG-3′ upstream primer with *Sac*I restriction site and primer 2 as downstream primer.

To prepare construct with ErbB-2 kinase domain and EGF receptor carboxyl terminus (residues 959–1186, according to Ullrich *et al.* (27)), *Sac*I restriction site in EGF receptor carboxyl terminus was mutated in 1 round PCR with primers 5′-CTC CTA AGT TCT CTG AGT GCA ACC-3′ (upstream primer) and 5′-TCA TAC TAT GGT GTC GAC TCA TGC TCC AAT AAA TTC ACT GCT TTG-3′ (downstream primer). PCR product was used in second round of PCR to generate EGF receptor carboxyl terminus with primer 5′-CAT TTG CCA AGT CCT CTA GAC TCC AAC TTC-3′ (upstream primer). The cDNA fragment for ErbB-2 kinase domain was prepared as described above with primers 1 and 3. Both products were cut with *Xba*I and ligated. Then cDNA fragment was cloned into pEGFP vector through *Sac*I and *Sal*I restriction sites. All the constructions described above were verified by sequencing in the regions that underwent genetic manipulations.

Immunoprecipitation and Immunoblotting—After indicated treatments, the cells were solubilized by scraping with rubber policeman into cold lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na_3VO_4). The lysates were then clarified by centrifugation (14,000 × *g*, 10 min). Receptors were immunoprecipitated with 1 μg of the indicated antibody immobilized on protein G or protein A by incubation for 1 h at 4 °C. Subsequently, the complexes were washed with lysis buffer three times and resuspended in Laemmli sample buffer for 7.5% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes, and the membranes were blocked by incubation with 5% bovine serum albumin in PBS for 1 h at room temperature. The membranes were then incubated 1 h at room temperature with the indicated blotting antibody in TBSTw buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20, 0.5% nonfat milk), washed three times in the same buffer, and incubated 1 h with horseradish peroxidase-conjugated mouse antibody or protein A. The membranes were then washed five times in TBSTw and visualized by ECL.

Confocal Microscopy—Cells were grown 1–2 days on Lab-Tek Chamber slides to 50–70% confluency, treated for indicated times with geldanamycin, and fixed by adding freshly prepared 4% paraformaldehyde in PBS and incubated for 1 h or overnight. Fixed SKBr3 and 3T3 cells were washed three times in PBS, permeabilized by incubation in 0.5% Triton X-100 in PBS for 10 min, and blocked by incubation with 5% bovine serum albumin in PBS for 1 h. Subsequently, the cells were incubated with fluorescene dye-conjugated secondary antibody for 1 h, washed five times, and dried. Fixed COS 7 cells expressing GFP fusion protein were observed directly.

RESULTS

Influence of ErbB-2 Kinase Domain on Geldanamycin-induced Degradation—To measure the extent of ErbB-2 degradation in cells treated with geldanamycin, we have used, in parallel, antibodies that react with ectodomain or carboxyl-terminal domain epitopes of ErbB-2. The results, as shown in Fig. 1*A*, demonstrate that immunoreactivity to both ectodomain and carboxyl-terminal domain antibodies is rapidly lost following exposure to geldanamycin. The cells employed in this experiment, SKBr3, overexpress ErbB-2, and quantitation of the data indicates that in these cells the half-life of ErbB-2 is approximately 2 h in the presence of geldanamycin. Others have reported a similar half-life for ErbB-2 under these conditions (19) and contrasts with the reported half-life of about 7–9 h for ErbB-2 under normal conditions (28, 29). This influence of geldanamycin on ErbB-2 metabolic stability is reasonably specific because no significant decrease in the structurally related receptors ErbB-1 or ErbB-4 was detected following geldanamycin treatment of A-431 cells or T47–17 cells, respectively (Fig. 1*B*).

To map the region of ErbB-2 that mediates geldanamycin sensitivity, we initially employed chimeric receptors in which the cytoplasmic domain of the EGF receptor is replaced by that of ErbB-2 (EGFR/ErbB-2^{CD}) or in which the carboxyl-terminal domain of the EGF receptor is replaced by the corresponding region of ErbB-2 (EGFR/ErbB-2^{CT}). These chimeric receptors are expressed in NIH 3T3 cells and have been described previously (25). The data in Fig. 2 show that following the addition of geldanamycin the chimeric receptor having the entire ErbB-2 cytoplasmic domain is sensitive to drug-induced degradation, whereas the chimeric receptor that contains only the carboxyl-terminal domain of ErbB-2 is not influenced by the presence of geldanamycin. In this experiment the EGFR/ErbB-2^{CD} receptor has a half-life of approximately 3.9 h in the pres-

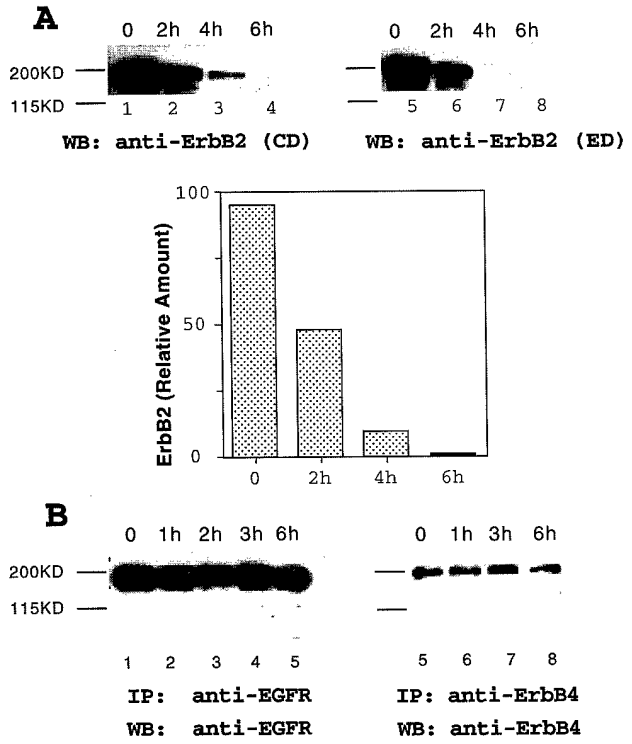

FIG. 1. **Metabolic stability of ErbB-2, EGF receptor, and ErbB-4 in the presence of geldanamycin.** *A*, SKBr3 cells were treated with geldanamycin (3 μM) for the indicated periods of time. The cells were then lysed, and Western blots (*WB*) were prepared from equal aliquots (50 μg) of the lysates using antibody to either the ErbB-2 cytoplasmic domain (*CD*) or ectodomain (*ED*). The blots in lanes 1–4 were evaluated by scanning densitometry to quantitate the amount of ErbB-2 present as a function of the time of drug exposure. *B*, A-431 cells that overexpress the EGF receptor (*lanes 1–5*) or NIH3T3 cells that overexpress a transfected human ErbB-4 receptor (*lanes 5–8*) were exposed to geldanamycin (3 μM) for the indicated periods of time. The cells were then lysed, and equal aliquots of each lysate (50 μg) were used for immunoprecipitation (*IP*) of the indicated receptor. Western blots were then performed as indicated, and bound antibody was visualized by ECL.

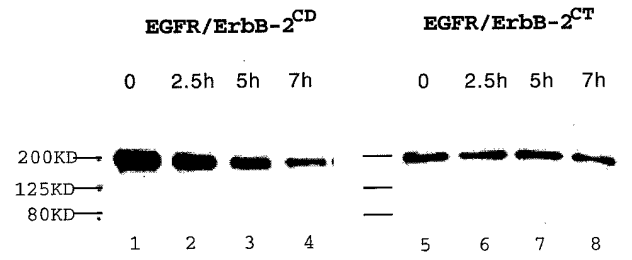

FIG. 2. **Evaluation of EGF receptor/ErbB-2 chimeric molecules for sensitivity to geldanamycin-induced degradation.** NIH3T3 cells expressing a chimeric EGFR with an ErbB-2 cytoplasmic domain (*EGFR/ErbB-2^{CD}*) or an ErbB-2 carboxyl-terminal domain (*EGFR/ErbB-2^{CT}*) were treated with geldanamycin (3 μM) for the indicated periods of time. The cells were lysed, proteins in equal aliquots (50 μg) of each lysate were separated by SDS-PAGE, and Western blots were performed using an antibody to an epitope in the carboxyl-terminal domain of ErbB-2. Bound antibody was visualized by ECL.

ence of geldanamycin. In the same background the ErbB-2 receptor has a half-life of about 3.3 h in the presence of this drug (data not shown). Therefore, the ectodomain of ErbB-2 does not have a significant role in determining the sensitivity of this receptor to geldanamycin-induced degradation. Given the data in Fig. 1*B* showing that the EGF receptor is not sensitive to geldanamycin, these results suggest that the kinase domain and/or juxtamembrane region of the ErbB-2 cytoplasmic domain mediate sensitivity to geldanamycin-induced degradation. This conclusion is consistent with the results of Miller *et al.* (19), who reported that an ErbB-2 internal deletion mutant lacking the kinase domain was not degraded in the presence of geldanamycin.

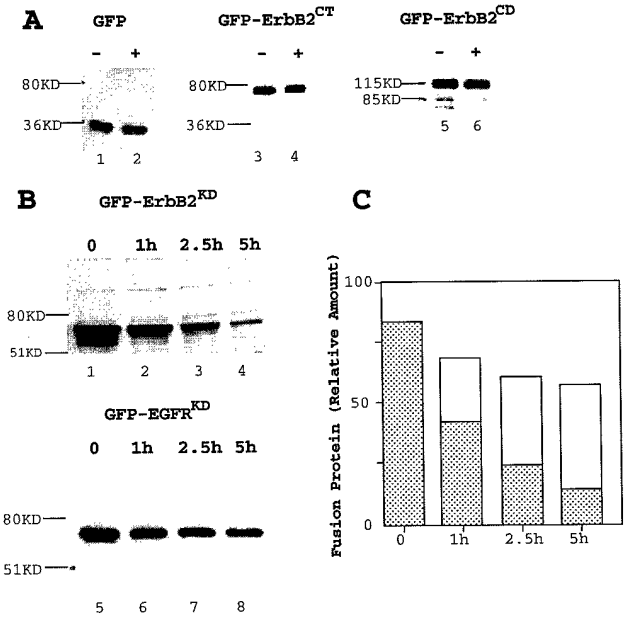

FIG. 3. **Sensitivity of GFP-ErbB2 fusion proteins to geldanamycin-induced degradation.** *A*, constructs encoding GFP, GFP-ErbB2^{CT}, or GFP-ErbB2^{CD} were expressed in COS 7 cells and tested for metabolic stability following incubation for 6 h in the absence (−) or presence (+) of geldanamycin (3 μM). Following the incubations, the cells were lysed, equivalent aliquots (30 μg) of each lysate were electrophoresed, and fusion proteins were detected by Western blotting with anti-GFP. *B*, constructs encoding GFP fusions with the ErbB-2 kinase domain (GFP-ErbB2^{KD}; *lanes 1–4*) or the EGF receptor kinase domain (GFP-EGFR^{KD}; *lanes 5–8*) were expressed in COS 7 cells. The cells were treated with geldanamycin (3 μM) for the indicated times prior to cell lysis. Equal aliquots (30 μg) of each lysate were then electrophoresed, and Western blotting with anti-GFP was used to detect fusion proteins. *C*, scanning densitometry of the data in *B* was used to quantitate the level of each fusion protein. Bound antibody was detected in each panel by ECL. *Closed bars* represent GFP-ErbB2^{KD}, and *open bars* represent GFP-EGFR^{KD}.

To directly test the possibility that the kinase domain determined geldanamycin sensitivity, we constructed GFP fusion proteins with the ErbB-2 cytoplasmic domain (GFP-ErbB-2^{CD}), the ErbB-2 kinase domain (GFP-ErbB-2^{KD}), the ErbB-2 carboxyl-terminal domain (GFP-ErbB-2^{CT}), or the EGF receptor kinase domain (GFP-EGFR^{KD}). These fusion proteins were expressed in COS 7 cells and tested for sensitivity to geldanamycin by blotting lysates with antibody to GFP after incubating the cells in the absence or presence of the drug. The results are shown in Fig. 3. As a control, we tested the sensitivity of GFP to geldanamycin, and, as shown in Fig. 3*A* (*lanes 1* and *2*), treatment with geldanamycin for 6 h induced no decrease in the cellular level of GFP. Also, there was no geldanamycin-induced degradation of the GFP fusion proteins containing the carboxyl terminus of ErbB-2 (Fig. 3*A*, *lanes 3* and *4*) or the entire cytoplasmic domain of ErbB-2 (Fig. 3*A*, *lanes 5* and *6*). However, the GFP fusion protein containing the ErbB-2 kinase domain (Fig. 3*B*, *lanes 1–4*) was rapidly degraded in the presence of geldanamycin, whereas there was no degradation of a fusion protein containing the EGF receptor kinase domain (Fig. 3B, *lanes 5–8*). The results in Fig. 3*B* are quantitated in Fig. 3*C* and show the increased sensitivity of the ErbB-2 kinase domain to geldanamycin compared with the EGF receptor kinase domain.

In this system, the lack of sensitivity to geldanamycin is, on

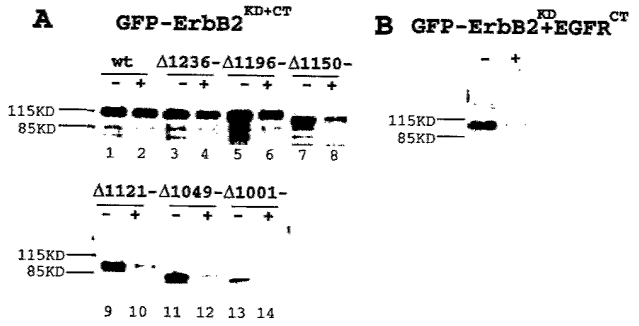

FIG. 4. **Role of the ErbB-2 carboxyl terminus in the sensitivity of the ErbB-2 kinase domain to geldanamycin-induced degradation.** *A*, constructs encoding fusion proteins of GFP with the ErbB-2 kinase and carboxyl-terminal domains (GFP-ErbB2^{KD+CT}) were expressed in COS 6 cells. The constructs contained the entire carboxyl-terminal domain (*wt*, *lanes 1* and *2*) or were truncated at the indicated residues in the carboxyl-terminal domain (*lanes 3–14*). The cells were then treated with (+) or without (−) geldanamycin (3 μM) for 6 h. Lysates were prepared, and equal aliquots (30 μg) were electrophoresed on 10% SDS-PAGE gels. Subsequently, Western blotting with anti-GFP was used to detect the fusion protein bands, which were visualized by ECL. *B*, a construct encoding a fusion protein of GFP with the ErbB-2 kinase domain and the EGF receptor carboxyl terminus (GFP-ErbB2^{KD} EGFR^{CT}) was expressed in COS 7 cells. The cells were then treated with (+) and without (−) geldanamycin (3 μM) for 6 h, and lysates were prepared. Equal aliquots (30 μg) of each lysate were electrophoresed and blotted with antibody to GFP. Bands were visualized by ECL.

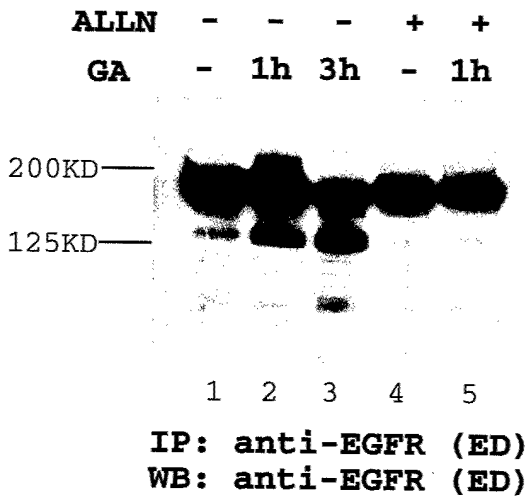

FIG. 5. **Evidence for fragmentation of ErbB-2 during geldanamycin** (*GA*)-induced degradation. NIH3T3 cells that express the chimeric EGF receptor containing the ErbB-2 cytoplasmic domain (EGFR/ErbB-2^{CD}) were preincubated for 1 h with ALLN (250 μM) as indicated prior to the addition of geldanamycin (3 μM) for the indicated periods of time. The cells were then lysed, and equal aliquots (0.5 mg) of each lysate were precipitated with antibody to the EGF receptor ectodomain (*EGFR(ED)*). Following SDS-PAGE, Western blotting with a second antibody to the EGF receptor ectodomain was used to detected immunoreactive bands, which were visualized ECL. *IP*, immunoprecipitation; *WB*, Western blot.

the basis of previous data as expected for fusion proteins containing the ErbB-2 carboxyl-terminal domain or the EGF receptor kinase domain. That the fusion protein containing only the ErbB-2 kinase domain is rapidly degraded in the presence of geldanamycin indicates that the kinase domain is sufficient to mediate its degradation. However, the lack of degradation of the fusion protein containing the entire ErbB-2 cytoplasmic domain was unexpected and is in apparent discordance with the geldanamycin-induced degradation of the EGF receptor/ErbB-2 chimeric receptor, which contains the entire ErbB-2 cytoplasmic domain (Fig. 2, *lanes 1–4*).

These latter results suggested that perhaps within the context of cytosolic GFP fusion proteins, but not in the transmembrane ErbB-2 molecule, the ErbB-2 carboxyl terminus had a protective effect on the sensitivity of the kinase domain to degradation induced by geldanamycin. To test this possibility we constructed a series of GFP fusion proteins containing the ErbB-2 kinase and carboxyl-terminal domains with progressive deletions of the ErbB-2 carboxyl-terminal domain. The geldanamycin sensitivity of these constructs, when expressed in COS 7 cells, is shown in Fig. 4. Deletion of the carboxyl-terminal 19 residues (Δ1236–1255, lanes *3* and *4*) or 59 residues (Δ1196–1255, lanes 5, 6) did not increase sensitivity of the fusion proteins to degradation in the presence of geldanamycin. However, increased sensitivity to geldanamycin-induced degradation was observed when deletions of 105 or more residues were made in the ErbB-2 carboxyl-terminal domain (*lanes 7–14*). Hence, loss of the amino-terminal half of this carboxyl-terminal domain significantly increases the sensitivity of the ErbB-2 kinase domain to geldanamycin.

In this series of fusion proteins we also determined whether the carboxyl-terminal domains of the EGF receptor would abrogate sensitivity of the ErbB-2 kinase domain to geldanamycin. Hence, we prepared a fusion protein construct to encode the kinase domain of ErbB-2 and carboxyl-terminal domain of the EGF receptor. When this molecule was expressed in COS 7 cells, the results (Fig. 4*B*) showed complete sensitivity to geldanamycin-induced degradation in contrast to the fusion protein containing the carboxyl-terminal sequences of ErbB-2 (*lanes 1* and *2*).

Detection of Geldanamycin-induced ErbB-2 Fragments—The above results, which indicate a role of the ErbB-2 carboxyl-terminal domain in determining the sensitivity of ErbB-2 kinase domain to geldanamycin-induced degradation, suggested that perhaps the carboxyl-terminal region of the transmembrane receptor might be cleaved following the addition of geldanamycin and that this event may be necessary for subsequent degradation of the ErbB-2 molecule, perhaps in a manner that required localization at the plasma membrane and not in the cytosol. Therefore, we re-examined the geldanamycin-induced degradation of the chimeric EGF receptor/ErbB-2^{CD} molecule, whose degradation in the presence of geldanamycin was shown in Fig. 2 (*lanes 1–4*). If the carboxyl terminus is, in fact, cleaved prior to degradation, then the antibodies used in the experiment shown in Fig. 2 would not detect the remaining membrane-localized fragment because they are to an epitope in the ErbB-2 carboxyl terminus. Hence, we tested the geldanamycin-induced degradation of this molecule using an antibody to the EGF receptor ectodomain. As shown in Fig. 5, this antibody detected both the native 185-kDa form of the chimeric receptor plus a geldanamycin-induced fragment of approximately 135 kDa (*lanes 2* and *3*). As shown in *lane 5*, the formation of this fragment in cells exposed to geldanamycin was blocked by the presence of the protease inhibitor ALLN and to a lesser extent by lactacystin (data not shown).

We next attempted to confirm that geldanamycin induced a carboxyl-terminal cleavage in the native ErbB-2 molecule as well as the chimeric receptor. To test this we used SKBr3 cells and an antibody to an epitope in the ErbB-2 ectodomain. As shown in Fig. 6*A* (*lane 2*) incubation of these cells with geldanamycin for 6 h resulted in the loss of the native ErbB-2 molecule, and no fragment was detected. This result is similar to that reported in Fig. 1*A* (*lanes 5–8*). We reasoned that perhaps in these cells the fragment was metabolically unstable and might be detectable if the geldanamycin treatment were performed at a low temperature to reduce metabolic degradation. In this part of the experiment (*lanes 3* and *4*), geldanamycin was added to the cells for 1 h at 37 °C, and then the cells were cooled to 4 °C and incubated for an additional 5 h. Under these conditions an

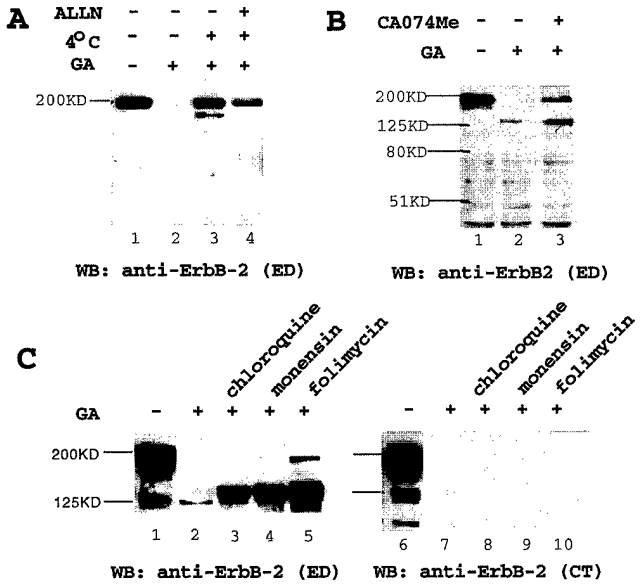

FIG. 6. **Geldanamycin (*GA*)-induced cleavage of ErbB-2.** *A*, as indicated, SKBr3 cells were preincubated with ALLN (250 μM) for 1 h. The cells were then incubated with geldanamycin (3 μM) for 5 h at 37 °C (*lanes 1* and *2*) or 1 h at 37 °C followed by 4 h at 4 °C (*lanes 3* and *4*). Cell lysates were then prepared, and equal (30 μg) aliquots of each lysate were analyzed by electrophoresis and Western blotting, using antibody to an epitope in the ErbB-2 ectodomain (*anti-ErbB-2 (ED)*) and ECL. *B*, SKBr3 cells were preincubated for 1 h with the cathepsin B inhibitor CA074-Me (100 μM) as indicated, and then geldanamycin (3 μM) was added for 6 h. Following cell lysis, aliquots (30 μg) of each lysate were electrophoresed and blotted with antibody to the ErbB-2 ectodomain (*lanes 1–3*) or antibody to the ErbB-2 carboxyl-terminal domain (*lanes 4–6*). Bound antibody was then visualized by ECL. *C*, SKBr3 cells were preincubated for 1 h as indicated with chloroquine (100 μM), monensin (50 μM), or folimycin (1.0 μg/ml). Geldanamycin (3 μM) was then added, and the incubation was continued an additional 6 h. Cell lysates were then prepared, and equal aliquots (30 μg) of each lysate were electrophoresed and Western blotted (*WB*) with antibody to the ectodomain of ErbB-2 (*lanes 1–6*) or antibody to the carboxyl terminus of ErbB-2 (*lanes 7–11*).Bound antibody was visualized by ECL.

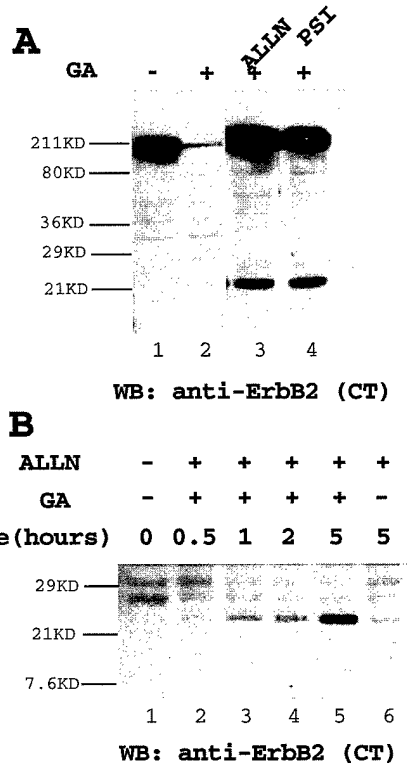

FIG. 7. **Detection of an ErbB-2 carboxyl-terminal fragment produced by exposure to geldanamycin (*GA*).** *A*, SKBr3 cells were preincubated for 1 h with ALLN (250 μM) or proteasome inhibitor I (100 μM), as indicated, prior to the addition of geldanamycin (3 μM) for an additional 6 h. *B*, SKBr3 cells were preincubated with ALLN (250 μM) for 1 h and then with geldanamycin (3 μM) for the indicated times. In both experiments cell lysates were prepared, and equal aliquots (30 μg) were subjected to electrophoresis and Western blotting (*WB*) using antibody to an ErbB-2 carboxyl-terminal domain epitope. Bound antibody was visualized by ECL.

ErbB-2 fragment of 135 kDa was detectable, and in the presence of ALLN this fragment was not detected. These data indicated that endogenous ErbB-2 is cleaved within the cytoplasmic domain in geldanamycin-treated cells.

A number of protease inhibitors were tested without success for their capacity to prevent degradation of the 135-kDa ErbB-2 fragment produced in response to geldanamycin. However, the cathepsin B inhibitor CA074-Me (30) did stabilize the level of the ErbB-2 fragment in geldanamycin-treated cells (Fig. 6*B*). Because cathepsin B is mainly localized in late endosomes (31), we tested compounds that interfere in the acidification and/or processing of endosomes. As shown in Fig. 6*C* (*lanes 1–5*), chloroquine, monensin, and folimycin each significantly increased accumulation of the 135-kDa ErbB-2 fragment in geldanamycin-treated cells. In the absence of geldanamycin, none of these compounds revealed major immunoreactive bands other than the native ErbB-2. Detection of this accumulated 135-kDa fragment was not possible when an antibody to the ErbB-2 carboxyl-terminal domain was employed (*lanes 6–10*), supporting the conclusion that this fragment is produced by a cleavage at the carboxyl terminus of ErbB-2.

If ErbB-2 is cleaved at the carboxyl terminus such that an antibody epitope is lost from the native molecule, an antibody to a carboxyl-terminal epitope may be able to detect the released fragment if it is sufficiently metabolically stable. That such a carboxyl-terminal ErbB-2 fragment can be detected in lysates from geldanamycin-treated cells blotted with an antibody to the ErbB-2 carboxyl terminus is shown in Fig. 7. As demonstrated in Fig. 7*A*, the presence of protease inhibitors ALLN or proteasome inhibitor I reveals the presence of a 23-kDa ErbB-2 carboxyl-terminal fragment produced during geldanamycin exposure of SKBr3 cells. The data in Fig. 7*B* show the influence of incubation time in geldanamycin on the accumulation of this fragment in SKBr3 cells. The fragment is readily detected within 1 h following the addition of geldanamycin. The previously described 135-kDa ErbB-2 fragment is also readily detected in the same period of time (data not shown).

Geldanamycin-induced Intracellular ErbB-2 Containing Vesicles—The capacity of folimycin, chloroquine, or monensin to increase accumulation of the 135-kDa ErbB-2 fragment suggests that this fragment is normally degraded by a mechanism that involves endocytic vesicles. Therefore, we used confocal microscopy to determine whether ErbB-2 is internalized during geldanamycin treatment. Following drug or vehicle exposure for 6 h, the cells were fixed and permeabilized prior to incubation with antibody to ErbB-2 and fluorescene-conjugated second antibody. Shown in Fig. 8 are SKBr3 cells with endogenous ErbB-2 (Fig. 8, *A* and *B*), NIH 3T3 cells stably expressing transfected ErbB-2 (Fig. 8, *C* and *D*), and COS 7 cells transiently expressing a GFP fusion protein with the ErbB-2 kinase domain (Fig. 8, *E* and *F*). In the absence of geldanamycin, the transmembrane form of ErbB-2 is clearly expressed at the cell surface in SKBr3 (Fig. 8*A*) and NIH 3T3 cells (Fig. 8*C*), whereas the GFP fusion protein is located in the cytosol of COS 7 cells (Fig. 8E). In each case the distribution is changed dramatically following geldanamycin incubation, such that immunoreactivity is concentrated in intracellular vesicles, which

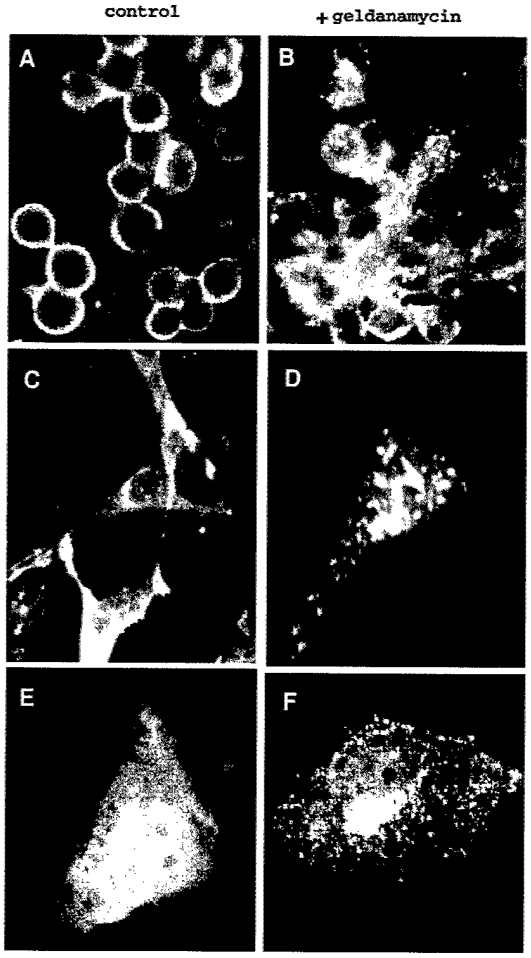

FIG. 8. **Formation of intracellular vesicles containing ErbB-2 during incubation with geldanamycin.** SKBr3 cells (*A* and *B*) or NIH3T3 cells expressing ErbB-2 (*C* and *D*) or COS 7 cells expressing the fusion protein GFP-ErbB2^{KD} (*E* and *F*) were growth on Lab-Tek slides. The SKBr3 and NIH3T3 cells were then incubated in the presence or absence of geldanamycin (3 μM) for 6 h. The COS 7 cells were incubated with or without geldanamycin for 3 h at 48 h post-transfection with the cDNA vector encoding GFP-ErbB2^{KD}. All cells were then fixed in paraformaldehyde, permeabilized with Triton X-100, and stained with antibody to the ErbB-2 ectodomain (*A–D*). Antibodies or GFP were visualized by confocal microscopy as described under "Experimental Procedures."

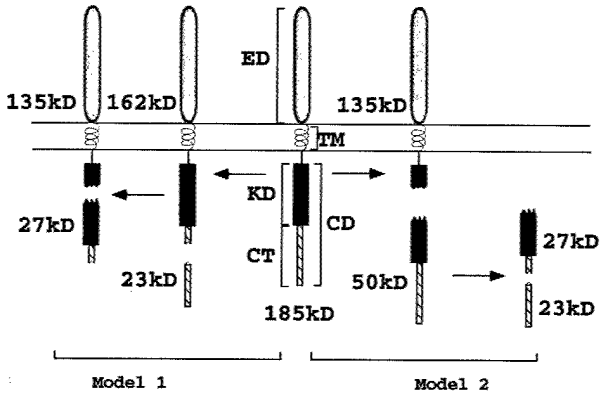

FIG. 9. **Schematic models for geldanamycin-dependent proteolytic fragmentation of ErbB-2.** In the center the 185-kDa native ErbB-2 molecule is depicted with ectodomain (*ED*), transmembrane domain (*TM*), kinase domain (*KD*), carboxyl-terminal domain (*CT*), and cytoplasmic domain (*CD*). Model I, to the *left*, depicts one potential route for ErbB-2 fragmentation, and model II, to the *right*, presents an alternate pathway.

mimic the appearance of lysosomes (Fig. 8, *B*, *D*, and *F*). The vesicles could be detected within 2 h of geldanamycin consistent with the time course of ErbB-2 degradation. The presence of either ALLN or folimycin decreased the redistribution of ErbB-2 immunoreactivity observed in the presence of geldanamycin (data not shown). Also, the GFP fusion protein with the entire ErbB-2 cytoplasmic domain, which we have previously shown is metabolically stable in the presence of geldanamycin (Fig. 3*A*, *lanes 5* and *6*), was not redistributed in the presence of the drug (data not shown).

DISCUSSION

In this manuscript we report several novel aspects of the mechanism by which geldanamycin induces the degradation of ErbB-2. Using antibodies to both ectodomain and carboxyl-terminal epitopes, we detect two fragments of ErbB-2 produced following geldanamycin incubation. One fragment of 135 kDa represents the ErbB-2 ectodomain plus the transmembrane domain and part of the kinase cytoplasmic domain. A second fragment of approximately 23 kDa is also detected under these conditions and represents the carboxyl-terminal domain of ErbB-2. These fragments are detectable within 30–60 min of geldanamycin addition to cells and continue to be detected for several hours. These two fragments do not, however, account for the entire mass of the native ErbB-2 molecule. In previous studies of geldanamycin-induced ErbB-2 degradation, fragments of this molecule have not been detected. This is likely due to two factors. The first is the absence of appropriate inhibitors and the second is the use of different antibodies for sequential precipitation (cytoplasmic domain epitope) and blotting (ectodomain epitope).

Based on the characteristics of these fragments, at least two mechanisms can be proposed to account for the generation of these fragments (Fig. 9). In model I, an endoproteolytic cleavage within the carboxyl-terminal domain could directly generate the observed 23-kDa fragment plus a 160-kDa fragment representing the rest of the ErbB-2 molecule. Subsequent cleavage of this latter fragment within the kinase domain could generate the observed 135-kDa fragment plus a small fragment of approximately 27 kDa that is undetectable with available immunologic reagents. Alternatively, it can be proposed that fragments are produced in the order predicted in model II. In this case an endoproteolytic event within the kinase domain would directly generate the observed 135-kDa transmembrane fragment plus a fragment of about 50 kDa. Subsequent cleavage of this latter fragment would produce the observed 23-kDa carboxyl-terminal domain fragment.

In either model intermediate fragments have not been detected (the 160-kDa fragment in model I or the 50-kDa fragment in model II), and this may be due to the rapidity with which the second cleavage occurs. We prefer the scheme depicted in model I for the following reasons. Our data show that expression of the ErbB-2 cytoplasmic domain as a cytosolic GFP fusion protein is not sensitive to geldanamycin-induced degradation, whereas a GFP fusion with the ErbB-2 kinase domain without the carboxyl-terminal domain is sensitive to geldanamycin-induced degradation. Also, the transmembrane ErbB-2 receptor is sensitive to degradation induced by geldanamycin. This suggests that proteolytic cleavage of the carboxyl-terminal domain may be restricted topologically within the cell to an area near the cytoplasmic face of the plasma membrane and not available to mediate cleavage of the cytosolic GFP fusion protein containing the entire ErbB-2 cytoplasmic domain. The exact protease(s) that generate these ErbB-2 fragments in response to geldanamycin have not been identified.

The metabolic stability of ErbB-2 in cells is likely complex, and ectodomain cleavage by metalloprotease activity has been

reported (32–35). In these experiments we have not observed ectodomain fragmentation, which produces fragments of different sizes than those we have detected in geldanamycin-treated cells.

The data in this manuscript indicate that complex series of proteolytic events are involved in the degradation of ErbB-2. This would include the proteosome as well as cathepsin B, an endosomal protease. Mimnaugh *et al.* (23) reported that lactacystin (10 μM), a proteosome inhibitor, blocked the geldanamycin-induced loss of the 185-kDa native form of ErbB-2. We find that lactacystin (10–40 μM) only partially prevents cleavage of the native ErbB-2 molecule and that ALLN is more effective in this regard. ALLN, like lactacystin, is a proteosome inhibitor, but it also inhibits other proteases such as calpain and cathepsin B and L. However, various calpain inhibitors (calpain inhibitor III, calpain inhibitor V, and calpastatin) do not block geldanamycin-induced fragmentation of ErbB-2 in our system.

In our experiments geldanamycin induces the formation of intracellular vesicles containing ErbB-2. Previously the accumulation of such vesicles was only reported after a prolonged 22-h incubation in the presence of geldanamycin and was attributed to the relocalization of newly synthesized ErbB-2 molecules (22). We observed the much more rapid formation of intracellular ErbB-2-containing vesicles, within 2 h of geldanamycin exposure. These vesicles also form when cells expressing the cytosolic GFP fusion protein with the ErbB-2 kinase domain are treated with geldanamycin. Also, the formation of these vesicles containing ErbB-2 is blocked by agents that interfere in the processing and acidification of endosomes, such as chloroquine, folimycin, and monensin. Hence, we conclude that these vesicles containing ErbB-2 are derived from the plasma membrane and that endosomal proteases, such as cathepsin B, participate in the degradation of internalized ErbB-2. The manner in which these vesicles are actually formed is, however, not clear. Inhibitors of cathepsin B, such as CA074-Me, have been shown to inhibited degradation of EGF and the EGF receptor within endosomes (30).

Lastly, our results indicate that the kinase domain of ErbB-2 mediates sensitivity to geldanamycin. Previously, Miller *et al.* (19) have shown that the ErbB-2 mutants lacking the entire cytoplasmic domain or the kinase domain are not degraded in cells treated with geldanamycin. Our analysis of chimeric receptors and GFP fusion protein agrees with that data.

Geldanamycin induces the metabolic degradation of other protein kinases, such as Raf and Src. In the case of Raf, in particular, the geldanamycin-binding protein Hsp90 along with another protein $p50^{cdc37}$ interact with the raf kinase domain (36, 37). It seems likely that a geldanamycin-binding protein, perhaps Hsp90, also interacts with the ErbB-2 kinase domain and, in the absence of geldanamycin, assists in the maintenance of this kinase domain in an active conformation. Given the intracellular abundance of proteins such as Hsp90, this interaction could be of low affinity and not readily detected by assays such as co-immunoprecipitation. Additional experiments will explore this issue.

Acknowledgments—We appreciate the assistance of Sue Carpenter in preparation of the manuscript, and we thank David Coon for technical assistance.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 36, Issue of September 7, pp. 33675–33680, 2001
Printed in U.S.A.

Caspase-dependent Cleavage of ErbB-2 by Geldanamycin and Staurosporin*

Received for publication, February 13, 2001, and in revised form, June 6, 2001
Published, JBC Papers in Press, June 11, 2001, DOI 10.1074/jbc.M101394200

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The geldanamycin-induced degradation of ErbB-2 produces a 23-kDa carboxyl-terminal fragment, which has been isolated and subjected to amino-terminal microsequencing. The obtained sequence indicates that the amino terminus of this fragment corresponds to Gly-1126 of ErbB-2. Analysis of the residues immediately before Gly-1126 suggests that cleavage may involve caspase activity. Site-directed mutagenesis of Asp-1125 in ErbB-2 prevents geldanamycin-provoked formation of the 23-kDa fragment, consistent with the requirement of this residue for caspase-dependent cleavage in known substrates. Also, the addition of the pan-caspase inhibitor Z-VAD-FMK blocks formation of the 23-kDa ErbB-2 fragment in cells exposed to geldanamycin. Interestingly, staurosporin and curcumin are also shown to provoke the degradation of ErbB-2 with formation of the 23-kDa carboxyl-terminal fragment. The generation of this fragment by staurosporin or curcumin is likewise blocked by caspase inhibition. Caspase inhibition does not prevent accelerated degradation of the 185-kDa native ErbB-2 in geldanamycin-treated cells but does significantly prevent staurosporin-stimulated metabolic loss of ErbB-2.

ErbB-2, a Type I transmembrane receptor tyrosine kinase, functions as a co-receptor by dimerizing with ligand-occupied members of the ErbB family, the EGF receptor, ErbB-3, or ErbB-4 (1–3). This heterodimerization event is considered to alter the signaling capacity and cellular responses provoked by homodimers of occupied ErbB receptors. No ligand has been identified that directly interacts with the ectodomain of ErbB-2.

ErbB-2 was originally identified as the transforming oncogene *neu*, which contains a point mutation in the transmembrane domain that is responsible for its oncogenic potential (4). Overexpression of ErbB-2 also produces a transformed phenotype in experimental systems (5–7). Importantly, ErbB-2 is frequently overexpressed in carcinomas, particularly mammary and ovarian carcinomas, and is associated with a poor prognosis (8–10). ErbB-2 antibodies (11, 12) and agents such as interferon (13) or tyrosine kinase inhibitors (14) decrease the growth of ErbB-2-expressing tumor cells and also reduce the cellular level of ErbB-2 (15). In many of these instances the decreased growth provoked by the loss of ErbB-2 is due to increased apoptosis. In contrast, the overexpression of ErbB-2 can prevent the induction of apoptosis (15). Hence, the growth-controlling activity of ErbB-2 is related to structural changes or alterations in its level of expression.

The benzoquinoid anasamycin antibiotic geldanamycin was first isolated and described as an inhibitor of tyrosine kinase activity (16). Subsequently, geldanamycin was shown to possess tumoricidal activity toward cell lines that overexpress ErbB-2 (17). Currently geldanamycin derivatives designed to reduce toxicity are in clinical trials for certain cancer patients (18). The addition of geldanamycin to cells results in an increased rate of degradation of several protein kinases, including ErbB-2 (17), Src (19), Raf (26), and focal adhesion kinase (20) as well as other growth-regulating proteins such as p53 (21). The mechanism by which geldanamycin provokes the degradation of these and other proteins is not clear but is thought to involve the Hsp90 family of chaperones, which are the major intracellular proteins that bind geldanamycin (22, 23). However, the manner in which Hsp90 or other geldanamycin-binding proteins influences the physiology of ErbB-2 is not known.

Geldanamycin binds to Hsp90 and inhibits its ATPase activity, which is required for its chaperone function (24, 25). Although association of Hsp90 with Src (19) and Raf (26) have been documented, only GRP94 was originally reported to be associated with ErbB-2 (27). The idea that GRP94 mediates geldanamycin-induced degradation of ErbB-2 was not widely accepted, however, due to its subcellular localization (31) and the fact that geldanamycin-sensitivity requires the tyrosine kinase domain of ErbB-2 (17, 29), which is never present within the lumen of the endoplasmic reticulum. However, recently it has been reported that Hsp90 does, in fact, associate with ErbB-2 in a manner that is disrupted by the presence of geldanamycin (30). Hence it is possible that Hsp90 is associated with the tyrosine kinase domain of ErbB-2 and may regulate the conformation or the enzymatic function of ErbB-2. Geldanamycin then represents a tool with which to perturb that association.

A previous study of ErbB-2 degradation stimulated by geldanamycin demonstrated the appearance of two fragments (29). One large fragment of 135 kDa included the ectodomain, the transmembrane domain, and part of the cytoplasmic domain. The other fragment of 23 kDa was derived from the carboxyl-terminal portion of the ErbB-2 cytoplasmic domain. These studies also indicated that the 135-kDa fragment was degraded by lysosomes, whereas degradation of the 23-kDa fragment depended on proteosome function. In the experiments reported in this manuscript, the exact nature of the 23-kDa fragment is described as is the nature of the enzyme that produces this fragment from ErbB-2.

* This work was supported by Department of the Army Breast Cancer Program Grant DAMD17-00-1-0483. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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EXPERIMENTAL PROCEDURES

Materials—Geldanamycin, curcumin, staurosporin, and enhanced chemiluminescence (ECL) reagents were purchased from Sigma. Polyvinylidene difluoride membrane was from Bio-Rad. *N*-Acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN),[1] caspase inhibitor I (Z-VAD-FMK), and proteasome inhibitor I were from Calbiochem. Monoclonal antibody against the ErbB-2 extracellular domain (Ab5) was from Transduction Laboratories, whereas monoclonal antibody against the ErbB-2 carboxyl-terminal domain (Ab3) was purchased from Calbiochem. The pcDNA3.1+ vector was obtained from Invitrogen. Goat anti-mouse antibody cross-linked with horseradish peroxidase was from Zymed Laboratories Inc., and LipofectAMINE was purchased from Life Technologies, Inc.

Cell Culture and Transfection—SKBr3 human breast cancer cells were grown to near confluence in 5% CO_2 at 37 °C in McCoy's medium with 10% fetal bovine serum and then washed and treated with the indicated compounds in Dulbecco's modified Eagle's medium. Cos7 cells were grown overnight in Dulbecco's modified Eagle's medium with 10% fetal bovine serum to about 80% confluence and then transfected with LipofectAMINE according to the manufacture's recommendations (3 μg of DNA of pcDNA3.1+ vector was used per 60-mm culture dish).

Construction of ErbB-2 Mutants—ErbB-2 mutants were prepared by site-directed mutagenesis using a megaprimer approach. To facilitate manipulations with the ErbB-2 intracellular region, a *Sal*I restriction site downstream of the transmembrane region was generated by polymerase chain reaction (silent mutagenesis) using the following primers: primer 1, ACT ACG TCC AGT TCT AGA TCA CAC TGG CAC GTC CAG ACC (3′); primer 2, AAG TAC ACG ATG CGT CGA CTG CTG CAG (5′); primer 3, CTG CAG CAG TCG ACG CAT CGT GTA(3′); primer 4, AGA CCC AAG CTG GCT AGC GCC ACC ATG GAG CTG GCG GCC TTG TGC CGC(5′). The resultant two polymerase chain reaction products (primers 1 and 2; primers 3 and 4) were cut with *Nhe*I, *Sal*I, and *Xba*I restriction sites and ligated into the pcDNA3.1+ vector through *Nhe*I and *Xba*I sites (3-piece ligation).

Mutations in the ErbB-2 carboxyl terminus were introduced in the first round of polymerase chain reaction with primer 1 as the 3′ primer and the following 5′ primers: primer 5, CTG CCC TCT GAG ACT GCT GGC TAC GTT (D1125-A); primer 6, TCT GAG ACT GAA GGC TAC GTT (D1125-E); primer 7, TCT GAG ACT GAT GCC TAC GTT GC (G1126-A). One strand of these products was used as the 3′ primer in the second round of polymerase chain reaction with primer 2 as the 5′ primer. The final products were ligated through *Sal*I and *Xba*I restriction sites into the pcDNA3.1+ vector. All mutations were confirmed by sequencing.

Immunoblotting—At the end of each experiment the cells were solubilized by scraping into cold lysis buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na_3VO_4). The lysates were then clarified by centrifugation (14,000 × *g*, 10 min), and equal aliquots (40 μg) were subjected to SDS-PAGE electrophoresis. Proteins were then transferred to a nitrocellulose membrane for Western blotting. The membrane was blocked by incubation with 5% bovine serum albumin in phosphate buffer saline for 1 h at room temperature. The membrane was then incubated for 1 h with the indicated primary antibody in TBSTw buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20, 0.2% nonfat milk), washed 3 times in the same buffer, and incubated for 1 h with horseradish peroxidase-conjugated second antibody. The membrane was then washed five times with TBSTw buffer and visualized by ECL.

Amino-terminal Microsequencing—SKBr3 cells were grown on 20 150-mm tissue culture dishes to 80% confluence (~10×10^6 cells/plate) and washed with Dulbecco's modified Eagle's medium. The cells were then preincubated for 1 h with ALLN (250 μM) and treated with geldanamycin (3 μM) for 5 h. Subsequently, the cells were scraped with a rubber policeman into cold lysis buffer and centrifuged (20,000 × *g*, 30 min). The high molecular weight proteins, including native ErbB-2, were removed by ultrafiltration using a Centriplus membrane with a cut-off of 100 kDa (Millipore). The filtrate (~30 ml) was incubated with 40 μg of antibody against ErbB-2 carboxyl terminus and protein G-Sepharose for 2 h and then washed five times with lysis buffer. Immunoreactive material was separated by 13% SDS-PAGE, transferred to the polyvinylidene difluoride membrane, and stained with Coomassie Blue. The 23-kDa band was cut out of the membrane and subjected to 7 cycles of Edman degradation using an Applied Biosystems Procise sequencer.

[1] The abbreviations used are: ALLN, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; PAGE, polyacrylamide gel electrophoresis.

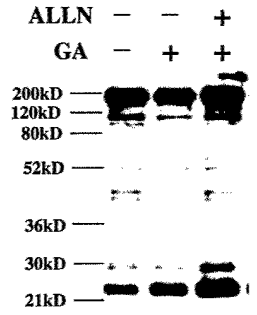

FIG. 1. **Geldanamycin-induced formation of the 23-kDa ErbB-2 fragment.** Cos7 cells were transfected with ErbB-2 as described under "Experimental Procedures." The cells were then treated with ALLN (250 μM) for 1 h, and geldanamycin (*GA*, 3 μM) was added for an additional 6 h. The cells were then lysed, and proteins in an aliquot of the lysate (40 μg) were separated by SDS-PAGE. A Western blot (*W.B.*) was performed using antibody against the ErbB-2 carboxyl-terminal domain (*CT*), and bound antibody was detected by ECL.

RESULTS

Sequencing of the 23-kDa Fragment of ErbB-2—Previously, we have shown that when the mammary carcinoma cell line SKBr3 is incubated with geldanamycin, a 23-kDa carboxyl-terminal fragment of ErbB-2 is produced (29). This fragment is rapidly degraded by proteosome activity unless a proteosome inhibitor is added. The data in Fig. 1 show that a similar 23-kDa fragment accumulates when transfected Cos7 transiently expressing human ErbB-2 are treated with geldanamycin and ALLN, a proteosome inhibitor.

To isolate this 23-kDa ErbB-2 fragment for sequence analysis, we employed transfected Cos7 cells treated with geldanamycin and ALLN for 6 h. ErbB-2 was then immunoprecipitated from cell lysates with an antibody to the carboxyl terminus of ErbB-2. The resulting immunoprecipitate was subjected to SDS-PAGE, the separated proteins were transferred to a polyvinylidene difluoride membrane, and the membrane was stained with Coomassie. The stained protein band at 23 kDa representing the carboxyl-terminal ErbB-2 fragment was clearly visible. The 23-kDa peptide was eluted from the membrane and subjected to automated Edman amino-terminal microsequencing, which yielded a sequence of GYVAPLT through the first seven cycles. There was no evidence of heterogeneity in this sequence, which if present might indicate proteolytic processing of the amino terminus of the fragment. The recovered sequence corresponds exactly to residues 1126–1132 in the deduced cDNA sequence of human ErbB-2 (32).

Caspase Cleavage of ErbB-2—Although it is possible that post-cleavage amino-terminal processing has taken place to yield the isolated 23-kDa fragment, we considered whether sequence information would predict a known proteolytic cleavage consensus site between residues 1125 and 1126 in ErbB-2. This analysis did show that there is a significant similarity between the ErbB-2 sequence immediately upstream from Gly-1126, which is PSETD, and caspase consensus cleavage sites that have been defined using a positional scanning combinatorial substrate library (33–35) and analysis of known caspase cleavage sites in proteins (36, 37). These caspase cleavage sequences are characterized by an essential Asp residue at the P1 position and frequently a Glu residue at the P3 position which correspond, respectively, to Asp-1125 and Glu-1123 in the ErbB-2 sequence.

To test the possibility that this putative caspase cleavage sequence in ErbB-2 was necessary to generate the 23-kDa

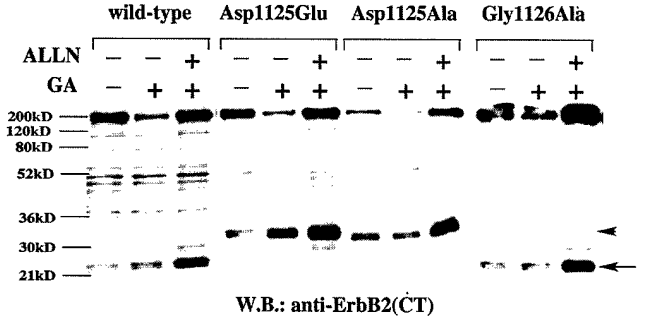

FIG. 2. **Influence of mutagenesis on formation of the 23-kDa ErbB-2 fragment.** Constructs encoding ErbB-2 wild type and mutants D1125A, D1125E, and G1126A were prepared as described under "Experimental Procedures" and expressed in Cos7 cells. Subsequently, the cells were preincubated for 1 h with ALLN (250 μM) before incubation with geldanamycin (*GA*, 3 μM) for an additional 6 h. The cells were then lysed, and an aliquot (25 μg) of each lysate was electrophoresed on a 12% SDS-PAGE. Immunoreactive bands were detected by Western blotting (*W.B.*) with an antibody against the ErbB-2 carboxyl-terminal domain (*CT*) and ECL. An *arrow* indicates the 23-kDa ErbB-2 fragment, whereas an *arrowhead* indicates the 30-kDa ErbB-2 fragment.

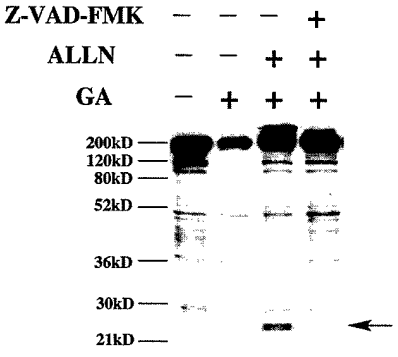

FIG. 3. **Influence of caspase inhibitor on formation of the 23-kDa ErbB-2 fragment.** SKBr3 cells were preincubated for 1 h with ALLN (250 μM) and Z-VAD-FMK (50 μM). The cells were then treated with (+) or without (−) geldanamycin (*GA*, 3 μM) for 6 h. Cell lysates were prepared and analyzed using 12% SDS-PAGE and Western blotting (*W.B.*) with antibodies against the carboxyl-terminal domain (*CT*). Bound antibody was visualized by ECL. An *arrow* denotes the 23-kDa ErbB-2 fragment.

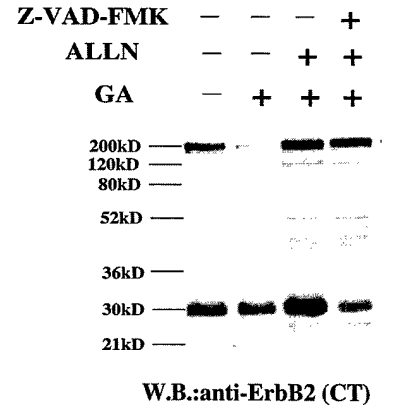

FIG. 4. **Influence of caspase inhibitor on formation of the 30-kDa ErbB-2 fragment.** The ErbB-2 mutant D1125A was expressed in Cos7 cells. The cells were then preincubated with ALLN (250 μM) for 1 h and with geldanamycin (*GA*, 3 μM) for an additional 6 h. Cell lysates (40 μg) were prepared and analyzed by SDS-PAGE and Western blotting (*W.B.*) with antibody against carboxyl-terminal domain (*CT*) of ErbB-2. Bound antibody was detected by ECL. An *arrowhead* indicates the 30-kDa band.

ErbB-2 fragment, site-directed mutagenesis was employed. The Asp at residue 1125 of ErbB-2 was mutated to either Ala or Glu, and Gly-1126 was changed to Ala. In regard to the conservative D1125E mutation, published structural data show that the aspartyl carboxylate side chain at the P1 position in a caspase substrate fits into a highly restrictive "socket" of enzyme that does not accommodate the side chain of a glutamate residue (36). The wild-type and mutant ErbB-2 constructs were transiently expressed in Cos7 cells and exposed to geldanamycin without or with ALLN. The results in Fig. 2 show that mutagenesis of Asp-1125 to either Glu or Ala prevented production of the 23-kDa fragment from ErbB-2. Interestingly, in the case of these two mutations a new fragment of 30 kDa was generated from the carboxyl terminus of ErbB-2, and the depletion of the cellular level of native ErbB-2 by geldanamycin was not affected. In the case of the G1126A mutation, there was no effect on geldanamycin-induced ErbB-2 degradation or formation of the 23-kDa fragment. That mutagenesis of Gly-1126 did not abrogate production of the 23-kDa fragment is not surprising in view of the fact that caspase cleavage sites, although preferring Gly at the P′1 position, exhibit considerable flexibility at this position (35).

To test the possibility that caspase activity is required for production of this 23-kDa fragment from ErbB-2, the pan-caspase inhibitor Z-VAD-FMK (38, 39) was tested. As can be seen in the data presented in Fig. 3, Z-VAD-FMK completely prevented the formation of the 23-kDa ErbB-2 fragment induced by geldanamycin. In the D1125E ErbB-2 mutant geldanamycin induces a 30-kDa fragment instead of the 23-kDa fragment. To determine whether the formation of this 30-kDa fragment might also be caspase-dependent, we incubated cells expressing the D1125E mutant with geldanamycin in the presence and absence of Z-VAD-FMK. The data shown in Fig. 4 indicate that the amount of the 30-kDa fragment is significantly reduced when these cells are exposed to geldanamycin in the presence of the caspase inhibitor.

ErbB-2 Degradation by Staurosporin—Geldanamycin induces ErbB-2 degradation, in part through a caspase-dependent mechanism, and geldanamycin is known to induce apoptosis in some cell lines (40). Therefore, we tested whether other reported inducers of apoptosis, particularly in mammary carcinoma cell lines, also provoke ErbB-2 degradation. Two known stimulators of apoptosis, which are also known protein kinase inhibitors, did induce ErbB-2 degradation in a manner similar to geldanamycin.

The results with staurosporin are shown in Fig. 5. The data in the *upper panel* of Fig. 5*A* show that staurosporin decreases the level of native ErbB-2 with about the same time course as that previously reported for geldanamycin (17), whereas the results in the *upper panel* of Fig. 5*B* show that maximal degradation of ErbB-2 was achieved with a staurosporin concentration of ~2 μM. Significantly, the ErbB-2 degradation elicited by staurosporin is accompanied by the production of a 23-kDa carboxyl-terminal ErbB-2 fragment, shown in the *lower panels* of Figs. 5, *A* and *B*, similar to that recorded for degradation induced by geldanamycin. The data in Fig. 6 demonstrate that formation of this 23-kDa fragment from ErbB-2 in the presence of staurosporin is prevented by the pan-caspase inhibitor Z-VAD-FMK. Comparison of geldanamycin and staurosporin-induced ErbB-2 degradation in the same cell line (SKBr3) can be made by comparing the data in Figs. 3 and 6, respectively. The results show that in the absence of ALLN, the 23-kDa fragment produced by staurosporin accumulates to a significantly greater level than the same fragment generated in geldanamycin-treated cells. Also, the presence of ALLN significantly increases the level of this fragment in geldanamycin-treated cells but actually decreases the fragment level in staurosporin-

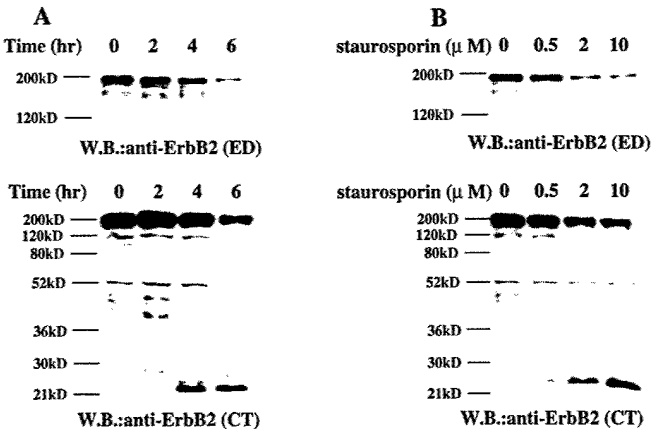

FIG. 5. **Staurosporin-induced degradation of ErbB-2.** SKBr3 cells were incubated with 5 μM staurosporin for the indicated times (*panel A*) or with the indicated concentrations of staurosporin for 6 h (*panel B*). Cell lysates were prepared, and aliquots (40 μg) were analyzed on 7.5% (*upper panels*) or 12% (*lower panels*) SDS-PAGE. Western blotting (*W.B.*) was then performed to detect the native ErbB-2 molecule using an antibody to the ErbB-2 ectodomain (*ED*) in the *upper panels* or the 23-kDa ErbB-2 fragment using an antibody to the ErbB-2 carboxyl-terminal domain (*CT*) in the *lower panels*. Bound antibody was detected by ECL.

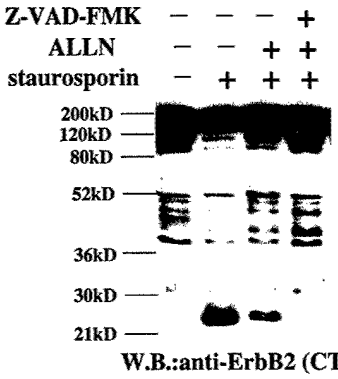

FIG. 6. **Capacity of caspase inhibitor to block the 23-kDa ErbB-2 fragment formation induced by staurosporin.** SKBr3 cells were preincubated with ALLN (250 μM) and Z-VAD-FMK (50 μM) for 1 h. The cells were then incubated with staurosporin (5 μM) for an additional 6 h. Cell lysates were prepared, and an aliquot (40 μg) of each was analyzed by SDS-PAGE and Western blotting (*W.B.*) with antibody against ErbB-2 carboxyl-terminal domain (*CT*). Bound antibody was detected by ECL.

treated cells. These results suggest significant, but unknown, distinctions in the mechanisms of action of geldanamycin and staurosporin.

Curcumin is another protein kinase inhibitor that has been reported to induce apoptosis in numerous cell lines and also to deplete cellular level of ErbB-2 (41–44). The data in Fig. 7 show that curcumin depletion of cellular ErbB-2 involves production of a 23-kDa ErbB-2 carboxyl-terminal fragment, which is stabilized in the presence of proteasome inhibitor I. Furthermore, the formation of this fragment in cells treated with curcumin is blocked in the presence of Z-VAD-FMK.

However, not all inducers of apoptosis provoked ErbB-2 degradation in SKBr3 cells. Etoposide (100 μM), actinomycin D (5 μg/ml), hydroxyurea (5 μM), camptothecin (100 μM), paclitaxel (1 μM) with or without the MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase) inhibitor PD98059 (50 μM) (45) and the potent kinase inhibitor H7 (100 μM) did not decrease ErbB-2 levels in SKBr3. In many cases, these compounds did induce dramatic morphological changes in the cells.

Both staurosporin and geldanamycin induce the degradation

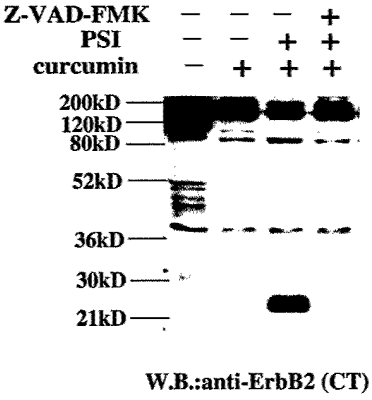

FIG. 7. **Curcumin-induced ErbB-2 degradation.** SKBr3 cells were preincubated as indicated with 75 μM proteasome inhibitor I (*PSI*) and Z-VAD-FMK (50 μM) for 1 h before the addition of curcumin (40 μM) for an additional 6 h. Cell lysates were prepared, and aliquots (40 μg) were subjected to the SDS-PAGE and Western blotting (*W.B.*) using antibody against the carboxyl-terminal domain (*CT*). Bound antibody was detected by ECL.

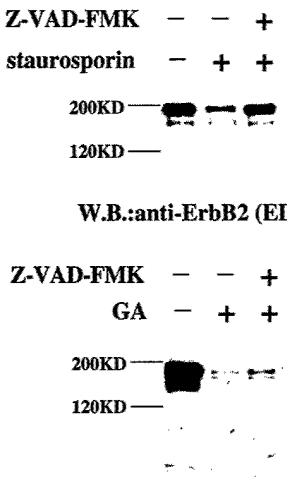

FIG. 8. **Caspase inhibitor prevents staurosporin- but not geldanamycin-induced degradation of ErbB-2.** SKBr3 cells were preincubated with Z-VAD-FMK (50 μM) for 1 h before the addition of staurosporin (5 μM) or geldanamycin (*GA*, 3 μM) for an additional 6 h. The cells were then lysed, and an aliquot (25 μg) of each lysate was electrophoresed and analyzed by Western blotting (*W.B.*) using antibody against ErbB-2 extracellular domain (*ED*). Bound antibody was detected by ECL.

of ErbB-2 with formation of a 23-kDa fragment that is dependent on caspase activity. We have asked whether the inhibition of caspase activity interferes with the drug-induced degradation of the native 185-kDa ErbB-2 molecule. The data in Fig. 8 show that Z-VAD-FMK does not block geldanamycin-induced ErbB-2 degradation, but the inhibitor significantly attenuates degradation induced by staurosporin. Like geldanamycin-induced ErbB-2 degradation, curcumin-mediated degradation of this transmembrane protein was not prevented by Z-VAD-FMK (data not shown).

DISCUSSION

The induction of ErbB-2 degradation by geldanamycin is associated with proteolytic fragmentation of the receptor (29). This includes the formation of a 23-kDa fragment that is recognized by antibodies as an epitope, within residues 1242–1255, in the carboxyl-terminal domain of the ErbB-2. Sequencing of this fragment shows that its amino terminus corresponds to Gly-1126 in the human ErbB-2 sequence. If the 23-kDa

fragment includes all residues from Gly-1126 to the ErbB-2 carboxyl terminus Val-1255, then it has a calculated molecular mass of 16,091. Hence its apparent M_r value on SDS-PAGE is an overestimation of the actual mass due to anomalous migration in the gel. This fragment would contain most of the known ErbB-2 autophosphorylation sites that provide its coupling to signal transduction proteins (46, 47) as well as the carboxyl-terminal PDZ domain recognition motif that is involved in targeting ErbB-2 to the basolateral surface of polarized cells (48).

It is possible that cleavage of ErbB-2 occurs between residues 1125 and 1126 to generate the 23-kDa fragment or that the primary cleavage event occurs upstream of these residues, and post-cleavage processing occurs at the amino terminus to produce the observed fragment. Several facts suggest that the former is more plausible. First, we did not detect heterogeneity in the amino-terminal amino acids recovered in microsequencing of this fragment. Second, the residues immediately upstream of Gly-1126 constitute a site favorable for caspase-dependent cleavage. This includes the essential Asp at residue 1125. Analysis of caspase cleavage sites shows that cleavage invariably occurs immediately after an Asp residue at the P1 position and frequently includes a Glu residue at the P3 position (34, 35), which in this instance corresponds to Glu-1123 in the ErbB-2 sequence. Our data show that mutagenesis of the essential Asp at position P1 (residue 1125) prevents geldanamycin-induced formation of the 23-kDa fragment. This includes the conservative D1125E mutation, which on the basis of the structure of caspase active sites with model substrates, is predicted to inhibit peptide bond hydrolysis by these proteases (36). Last, the pan-caspase inhibitor Z-VAD-FMK blocks geldanamycin-induced formation of the 23-kDa peptide. Hence, we conclude that this fragment is formed by cleavage between residues 1125 and 1126 in ErbB-2.

It is interesting that when Asp-1125 is mutated in ErbB-2, geldanamycin treatment of cells gives rise to a new carboxyl-terminal fragment of 30 kDa instead of the 23-kDa fragment. Formation of this fragment in the ErbB-2 mutant is partially sensitive to the caspase inhibitor. There are consensus caspase cleavage sites upstream of Asp-1125 that could generate the 30-kDa fragment. These include Asp-1087 and Asp-1016 and Asp-1019, which constitute a nested cleavage site. Cleavage at Asp-1087 would directly produce a carboxyl-terminal ErbB-2 fragment of about 30 kDa, whereas cleavage at Asp-1016 or Asp-1019 would generate a theoretical 40-kDa fragment, which would require additional processing to produce the observed 30-kDa fragment.

It is important to note that when the caspase-dependent cleavages at the carboxyl terminus are prevented by the addition of Z-VAD-FMK to geldanamycin-treated cells, the cellular content of the native ErbB-2 molecule is still decreased. This implies that cleavage at the ErbB-2 carboxyl terminus is not necessary to initiate the geldanamycin-dependent reduction in the ErbB-2 levels. However, Z-VAD-FMK does prevent staurosporin-dependent degradation of the native ErbB-2 molecule, suggesting that caspase activity is more significant in the degradation of ErbB-2 by staurosporin than by geldanamycin.

The current proposed mechanism for geldanamycin-induced ErbB-2 degradation involves the drug-dependent dissociation of Hsp90 from the kinase domain of ErbB-2 (27, 28, 30). Since geldanamycin is known to induce apoptosis and some but not all cell types (40, 49, 53) and, as mentioned above, caspase activity seems involved in ErbB-2 degradation induced by this drug, we tested other known inducers of apoptosis for their capacity to provoke ErbB-2 degradation and formation of the 23-kDa fragment. Of the various inducers of apoptosis tested, two compounds (staurosporin and curcumin) were found to promote formation of a 23-kDa fragment. Others report that curcumin inhibits the tyrosine kinase activity of ErbB-2 and depletes the cellular content of native ErbB-2 (41). Curcumin also induces an apoptotic response in mammary carcinoma cells (50). Curcumin has been reported to dissociate the endoplasmic reticulum heat shock protein GRP94 from ErbB-2 (41). However, it is not known whether curcumin actually binds to GRP94, and its effect on Hsp90 has not been reported.

Less is known regarding the effects of staurosporin on ErbB-2. Staurosporin, a broad spectrum protein kinase inhibitor, has not been reported to interact with heat shock proteins and is well described as an inducer of apoptosis (51, 52). Staurosporin, like geldanamycin and curcumin, is a weak inhibitor of receptor tyrosine kinases (51) but has not been previously shown to alter the cellular level of ErbB-2. The similarities in ErbB-2 metabolism between cells treated with geldanamycin or staurosporin suggest that the observed degradation of ErbB-2 by these compounds does not necessarily have to proceed through dissociation of heat shock proteins from ErbB-2. It may be that inhibition of kinase activities along with promotion of caspase activities is sufficient for ErbB-2 degradation. The identity of the target kinases, however, is unclear. It has been reported that the metabolic stability of a kinase-negative ErbB-2 mutant is decreased by geldanamycin (30), which suggests that perhaps non-tyrosine kinases are the target of these drugs.

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[CANCER RESEARCH 63, 39–43, January 1, 2003]

Identification of ErbB-2 Kinase Domain Motifs Required for Geldanamycin-induced Degradation[1]

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ABSTRACT

The ansamycin antibiotic geldanamycin (GA) induces the intracellular degradation of ErbB-2/*neu*. Degradation of ErbB-2 proceeds through cleavage(s) within the kinase domain, resulting in the formation of a 135 kDa ectodomain fragment and a fragment(s) of ~50 kDa containing the COOH-terminal region. On the basis of independent means of identification, two adjacent sequence motifs have been identified in ErbB-2 that are required for GA-induced degradation. These motifs encompass residues 776–783 and 784–786 within the NH_2-terminal lobe of the ErbB-2 kinase domain. This is also a region in which the epidermal growth factor receptor and ErbB-2 kinase domains differ significantly in sequence. Although mutations in this region abrogate GA-induced ErbB-2 degradation, the tyrosine kinase activity of ErbB-2 is not disrupted. Interestingly, these ErbB-2 mutants are specifically resistant to GA-induced degradation but retain sensitivity to other drugs, such as staurospore and curcumin, which are also able to provoke ErbB-2 degradation.

INTRODUCTION

ErbB-2, a member of the EGF[3] receptor family that includes four Type I receptor tyrosine kinases, is an orphan receptor that functions as a coreceptor through the formation of ligand-dependent heterodimers with other ErbB family members (1–3). Seven structurally related ligands (EGF, transforming growth factor-α, heparin-binding EGF, amphiregulin, betacellulin, epiregulin, and epigean) are recognized by the EGFR (ErbB-1), whereas the heregulins bind directly to the ErbB-3 and ErbB-4 receptors (2). In the case of ErbB-3, dimerization with ErbB-2 is essential because ErbB-3 does not have tyrosine kinase activity in its absence (4). Heterodimerization and signaling through ErbB-2 enhance the strong proliferative effect of all these growth factors (5–7).

Overexpression of ErbB-2 has been demonstrated in human breast, ovarian, prostate, and lung cancers. HER2 gene amplification is usually, but not always, responsible for a 10–100% increase in the ErbB-2 protein content of tumor cells, which results in constitutive activation of signaling pathways leading to uncontrolled proliferation, increased cell motility, invasiveness, and tumorigenesis (8, 9). ErbB-2 overexpression correlates with an aggressive disease form, particularly in the case of breast cancer, and a poor prognosis, making it a significant target for therapeutic intervention (10).

Monoclonal antibodies against the ErbB-2 ectodomain have been shown to be effective against tumors that overexpress ErbB-2 and are in use clinically (11–13). However, their mechanism of action is poorly understood but appears to involve the accelerated internalization and degradation of ErbB-2 (11). Another potential therapeutic approach is to use specific tyrosine kinase inhibitors that block ErbB-2 kinase activity and at the same time increase the rate of ErbB-2 degradation (14–16). ErbB-2 degradation is significantly increased in the presence of some nonselective tyrosine kinase inhibitors, such as GA or curcumin (17).

GA was first described as an inhibitor of tyrosine kinase activity. It belongs to the group of benzoquinoid ansamycin antibiotics and is closely related in structure to herbimycin A and macbecin (18). Ansamycin antibiotics were shown to have selective cytotoxicity against several malignant tumor cell lines, revert to normal the morphology of transformed fibroblasts, and reduce tumorigenicity in murine models. In cell lines, GA increases the rate of degradation of several protein kinases, including ErbB-2, Src family members, Raf-1, focal adhesion kinase, Met, and Bcr-Abl (19–24). Although the molecular mechanism of GA action is not clear, it is thought to bind to Hsp90 and inhibit the binding of this chaperone to protein kinases and thereby provoke the metabolic destabilization and degradation of Hsp90 targets (25, 26).

Previous studies have demonstrated that the ErbB-2 kinase domain is necessary for GA-induced degradation of this receptor (25, 27). It has been proposed that Hsp90 stabilizes ErbB-2 through interaction with its kinase domain, and this interaction is perturbed in the presence of GA. In this study, evidence is presented that the ErbB-2 kinase domain contains a cryptic motif that facilitates GA-induced receptor degradation.

MATERIALS AND METHODS

Materials. GA, curcumin, staurosporine, and ECL reagents were purchased from Sigma Chemical Co. (St. Louis, MO). ALLN and folimycin were from Calbiochem. Monoclonal antibody against ErbB-2 extracellular domain (Ab5) was from Transduction Laboratories, monoclonal antibody against ErbB-2 cytoplasmic domain (Ab3) was from Oncogene Science, monoclonal antibody against GFP was from Clontech, and polyhistidine antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The pcDNA3.1+ vector was obtained from Invitrogen. Goat antimouse antibody cross-linked with horseradish peroxidase was from Zymed, whereas LipofectAMINE was purchased from Life Technologies, Inc.

Cell Culture and Transfection. Human mammary SKBr3 cells were grown in 5% CO_2 at 37°C in McCoy medium with 10% FCS. Cos7 cells were grown in DMEM with 10% FCS. SKBr3 cells were grown to ~90% confluency and treated with the indicated drugs. For transient transfection experiments, Cos7 cells were grown to ~80% confluency and transfected with LipofectAMINE according to manufacturer's recommendations. The cells were then grown for 48 h before assays were conducted.

Construction of ErbB-2 Mutants. ErbB-2 mutants truncated at residues 718, 786, 788, 802, 808, 813, 823, and 990 were prepared by PCR. We used the pcDNA3.1 vector containing full-length ErbB-2. The cDNA contains a *Xho*I restriction site after the stop codon and a *Sac*II restriction site in the transmembrane domain, which was introduced by silent mutagenesis (codon TCT corresponding to residue 656 was replaced with TCC, and natural *Sac*II site downstream in the cytoplasmic region was removed also using silent mutagenesis). The cytoplasmic domain of ErbB-2 was excised using *Sac*II and *Xho*I restriction sites. Next, cytoplasmic domain segments of ErbB-2 mutants were prepared by PCR and ligated into a vector that contained ErbB-2 ectodomain through *Sac*II and *Xho*I restriction sites. Segments corresponding to the cytoplasmic domain of particular truncated mutants were amplified by PCR with 5′ primer 1 TGC ACC CAC TCC TGT GTG GAC CTG and 3′

Received 7/18/02; accepted 10/28/02.

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[1] Supported by the Department of Defense Idea Award Grant DAMD 17-00-1-0483.

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[3] The abbreviations used are: EGF, epidermal growth factor; GA, geldanamycin; ALLN, *N*-acetyl-L-leucinil-L-leucinil-L-norleucinal; ECL, enhanced chemiluminescence; GST, glutathione *S*-transferase; EGFR, epidermal growth factor receptor; GFP, green fluorescence protein; PLC, peritoneal lymphocyte.

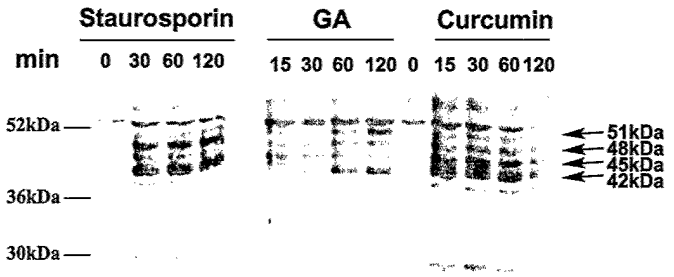

Fig. 1. Drug-dependent metabolic degradation of ErbB-2. SKBr3 cells were incubated with 3 μM GA, 5 μM staurosporine, or 40 μM curcumin for 6 h. The cells were then lysed, and equal aliquots were subjected to SDS-PAGE and Western blotting with antibody against the ErbB-2 COOH terminus (residues 1246–1255). Bound antibody was detected with ECL.

primers corresponding to the last seven residues of ErbB-2 before each truncation and containing *Xho*I restriction site after the stop codon.

Parts of the ErbB-2 or EGFR kinase domains fused to GFP were generated by PCR using the following primers: ErbB-2, 5′primer GGG ATC CTC ATC AAG C*GA GCT* CAG CAG AAG ATC, 3′ primers CAC GTA TGC TTC *GTC TAG A*AT TTC TTT, CCG CAC ATC *CTC TAG A*TA GCT CAT; EGFR 5′ primers GCC AAC AAG GAA AT*T CTA GA*T GAA GCC, AAG GGC ATG AAC TA*T CTA GA*G GAC CGT, 3′ primer GTA GAA GTT GGA *CTC GAG* AGG ACT TGG. PCR products were ligated through *Xba*I sites, reamplified using ErbB-2 5′ primer and EGFR 3′ primer, and inserted into pEGFP-C1 vector through *Sac*I and *Xho*I restriction sites.

Site-directed mutagenesis of ErbB-2 residues 813–817 NRGRL to HKDNI (mutant 3), 785–788 LLGI to AAGA (mutant 4), and GVGSPYVS to SVD-NPHVC (mutant 5) was done using the PCR megaprimer approach. In the first round of PCR, we used 5′primer 1 (above) and 3′primers ACA CCA GTT CAG CAG GTC CTG GGA GCC **AAT GTT GTC TTT** GT**G** TTC CCG GAC ATG GTC TAA GAG GCA GCC (mutant 3), TGT CAG GCA G**GC** GCC C**GC** A**GC** GCG GGA GAC (mutant 4), or CAG AAG GCG G**C**A CAC **GTG** TGG **GTT** GTC CAC AC**T** AGC CAT CAC (mutant 5). In the second round of PCR, we used the first-round product of PCR as megaprimer and primer ACT ACG TCC AGT *CTC GAG* TCA CAC TGG CAC GTC CAG ACC. PCR products from the second round were ligated through *Sac*II and *Xho*I sites into pcDNA3.1 containing the ErbB-2 without cytoplasmic domain, excised by *Sac*II and *Xho*I.

Immunoblotting. At the end of each experiment, the cells were solubilized by scraping into cold lysis buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM Na3VO4]. The lysates were then clarified by centrifugation (14,000 × *g* for 10 min), and aliquots containing equal amounts of protein were subjected to SDS-PAGE. Subsequently, proteins were transferred to nitrocellulose membranes, and the membrane was blocked by incubation with 5% BSA in PBS for 1 h at room temperature. The membrane was then incubated for 1 h with the indicated antibody in TBSTw buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20, 0.2% nonfat milk], washed three times in the same buffer, and incubated for 1 h with horseradish peroxidase-conjugated antimouse antibody. The membranes were then washed five times with TBSTw buffer and visualized by ECL.

Kinase Assay. Constructs of wild-type ErbB-2, the M4 or M5 mutants, were transiently transfected into Cos7 cells. After 48 h, cell lysates were prepared, and antibody against the ErbB-2 ectodomain was used to immunoprecipitate ErbB-2 or the mutants. Recombinant GST/PLC-γ1 SH2-SH2-SH3 fusion protein was used as a substrate in the *in vitro* kinase assay. Equal volumes of agarose beads containing equivalent amounts of protein, as confirmed by Western blotting, were incubated for 2 h with 20 μg of substrate in a kinase assay buffer (20 mM HEPES, 100 μM sodium orthovanadate, 1 mM DTT, 10 mM MgCl2, 10 mM MnCl2, and 20 μM ATP). Next, the PLC-γ1-derived substrate was precipitated with GST antibody and blotted with antibodies against phosphotyrosine or GST.

RESULTS

Drug-induced Cleavage within the ErbB-2 Kinase Domain. Several tyrosine kinase inhibitors, such as GA, curcumin, and staurosporine, induce ErbB-2 metabolic degradation and quickly deplete this receptor from cells (18, 28, 29). Previously, we reported that in the presence of GA, ErbB-2 is cleaved within its cytoplasmic domain (27). Cleavage in the ErbB-2 COOH-terminal domain by caspase activity is observed after 2–4 h incubation with drugs (29). In contrast, the data in Fig. 1 show that cleavage(s) within the ErbB-2 kinase domain is observed as early as 15–30 min after the addition of GA, curcumin, or staurosporine. In this experiment, an antibody against the ErbB-2 COOH-terminal residues 1246–1255 detected three to four fragments, of 42–51 kDa, induced by incubation of SKBr3 cells with each of these drugs. The relative molecular mass of these fragments combined with the location of the antibody epitope indicates that cleavage(s) occur close to or within the kinase domain. All three drugs induce formation of the 42, 48, and 51 kDa fragments, although the time course for the appearance of each fragment and the relative amount of each fragment are different. Previously, we have demonstrated that when ectodomain antibodies are used to detect GA-induced ErbB-2 fragments, a fragment of 135 kDa is detected, which includes the ecto- and transmembrane domains plus part of the cytoplasmic domain (27). Therefore, the combination of this fragment and the COOH-terminal fragments detected in Fig. 1 are sufficient to represent the result of a GA-induced cleavage of ErbB-2 within its kinase domain.

In previous studies, we *et al.* (18, 25, 27) established that the ErbB-2 kinase domain is necessary for GA-induced degradation of the receptor. The kinase domain is also sufficient for GA-induced degradation, *e.g.,* when a soluble fusion protein containing GFP and the ErbB-2 kinase domain is exposed to GA, the fusion protein is rapidly degraded (27). To extend this observation to the transmembrane ErbB-2 molecule, wild-type or ErbB-2 cytoplasmic domain truncation mutants were expressed in Cos7 cells. The cells were then incubated with GA or GA and ALLN, a protease inhibitor, which blocks GA-induced ErbB-2 degradation. The results in Fig. 2 show that an ErbB-2 mutant, truncated at residue 718 (containing the extracellular and transmembrane domains plus 42 residues of the cytoplasmic domain but not the kinase or COOH-terminal domains), has a high level of expression in Cos7 cells and is metabolically stable in the presence of GA. In contrast, a mutant truncated at the end of the kinase domain (*i.e.,* residue 990) and lacking only the COOH-terminal domain is unstable in Cos7 cells because of rapid basal degradation. Degradation of this mutant, however, can be enhanced by the presence of GA. In contrast to the behavior of these mutants, wild-type ErbB-2 expressed in Cos7 cells is constitutively stable, but its level is significantly decreased in the presence of GA.

These data indicate that the removal of the ErbB-2 COOH-terminal

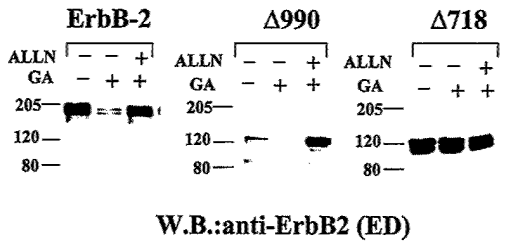

Fig. 2. Influence of truncation mutations on GA-induced ErbB-2 degradation. Constructs encoding ErbB-2 or ErbB-2 mutants truncated at residues 718 or 990 were expressed in Cos7 cells. At 48 h after transfection, the cells were treated with GA (3 μM) for 6 h, and cell lysates were prepared. Equal aliquots of each lysate were electrophoresed and blotted with antibody to ErbB-2 extracellular domain.

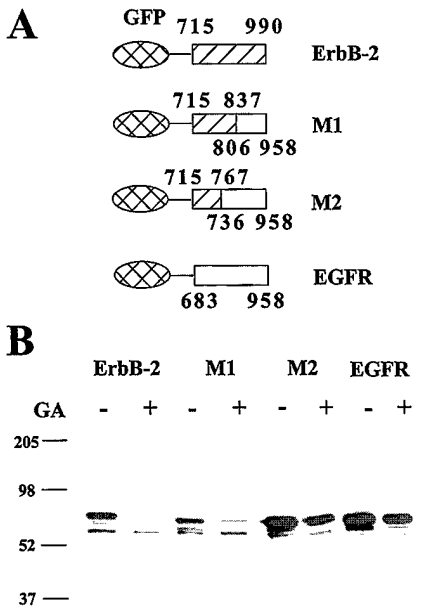

Fig. 3. Sensitivity of ErbB-2/EGFR kinase domain chimeric fusion proteins to GA-induced degradation. *A*, constructs used in experiment: GFP~ErbB-2 kinase domain (residues 679–999); mutant M1: GFP fused to chimeric kinase domain, which includes the NH_2-terminal part of ErbB-2 kinase domain (residues 679–837) and the COOH-terminal part of the EGFR kinase domain (residues 806–968); mutant M2: GFP fused to chimeric kinase domain, which includes the NH_2-terminal part of ErbB-2 kinase domain (residues 679–767) and COOH-terminal part of EGFR kinase domain (residues 806–968); GFP~EGFR kinase domain (651–968). Each construct also includes several residues beyond the kinase domain. In *B*, GFP fusion proteins were expressed in Cos7 cells. At 48 h after transfection, the cells were incubated with GA (3 μM) for 6 h. Equal aliquots of cell lysates were subjected to SDS-PAGE and Western blotting with antibody against GFP.

domain (residues 990-1255) destabilizes the molecule and decreases its basal level of expression. Further truncation to include the kinase and COOH-terminal domains (residues 718-1255) restores the basal metabolic stability of the truncated molecule but eliminates sensitivity to GA, suggesting that GA-induced degradation of ErbB-2 requires sequence information between residues 778 and 990.

The results in Fig. 2 are consistent with an experimental system we described previously in which the ErbB-2 kinase domain was fused to GFP. Expression of this construct was stable in Cos7 cells, but the addition of GA leads to its rapid degradation (27). In addition, we *et al.* (25, 27) have reported that the EGFR is considerably more stable than ErbB-2 in the presence of GA. Therefore, we made GFP fusion proteins containing the ErbB-2 kinase domain, the EGFR kinase domain, or chimeric kinase domains (termed mutants M1 and M2) containing NH_2-terminal portions of the ErbB-2 kinase domain fused to COOH-terminal portions of the EGFR kinase domain (Fig. 3*A*). These constructs were expressed in Cos7 cells and tested for their sensitivity to GA. The results show clearly that the ErbB-2 kinase is much more sensitive to GA than the EGFR kinase domain (Fig. 3*B*). These data also show that the kinase domain chimeric construct M1 is sensitive to GA, whereas the M2 chimera is resistant to GA. In the M1 mutant, residues 715–837 of the ErbB-2 kinase domain are followed by residues 806–958 of the EGFR kinase domain. In the M2 mutant, residues 715–767 of the ErbB-2 kinase domain are followed by residues 736–958 of the EGFR kinase domain. Both mutants contain a complete tyrosine kinase domain. These results indicate, therefore, that a motif located between residues 767 and 837 of the ErbB-2 kinase domain confers sensitivity to GA and is consistent with the data shown in Fig. 2 with ErbB-2 kinase domain truncation mutants.

Kinase Domain Motifs Required for GA Sensitivity. The preceding data suggest that determinants of GA-sensitive degradation are located within residues 767–837 of the ErbB-2 kinase domain. Within this region, ErbB-2 and the EGFR are highly homologous with the exception of two multiresidue motifs. When residues 776–783 of ErbB-2 are compared with the corresponding residues of the EGFR, the sequences differ at five of eight residues. In addition, residues 813–817 of ErbB-2 differ from the corresponding residues of the EGFR at five of five positions. Therefore, we used mutagenesis to change the sequence of ErbB-2 to that of the EGFR at each of these two locations.

When the sequence in ErbB-2 between residues 813 and 817 (NRGRL) was changed to that of the EGFR (HKDNI) and the mutant protein (termed M3) was expressed, it remained as sensitive to GA as the wild-type ErbB2 (Fig. 4). However, alteration of the ErbB-2 sequence between residues 776 and 783 (GVGSPYVS) to that present in the corresponding position of the EGFR (SVDNPHVC) had a significant influence on GA-induced ErbB-2 degradation. As shown in Fig. 4, this mutation (termed M4) prevented GA-induced degradation of ErbB-2.

The data in Fig. 2 not only indicate that sensitivity to GA in ErbB-2 is mediated by sequence information between residues 718 and 990 of the kinase domain but also show that the basal metabolic stability (*i.e.*, in the absence of GA) influences the same sequence information. Therefore, we constructed a series of ErbB-2 truncation mutants that encompass this region. Beginning at residue 718, successive mutations were made through residue 823 as depicted in Fig. 5. The basal

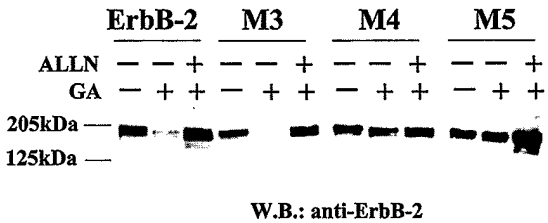

Fig. 4. Influence of mutations in the ErbB-2 kinase domain on GA-induced degradation. Wild-type ErbB-2 and ErbB-2 mutants 3–5 were each expressed in Cos7 cells. Approximately 48 h later, the cells were preincubated for 1 h with ALLN (250 μM) before the addition of GA (3 μM) for 6 h., as indicated. Equal aliquots of cell lysates were subjected to SDS-PAGE. Western blotting was performed with antibody against the ErbB-2 extracellular domain.

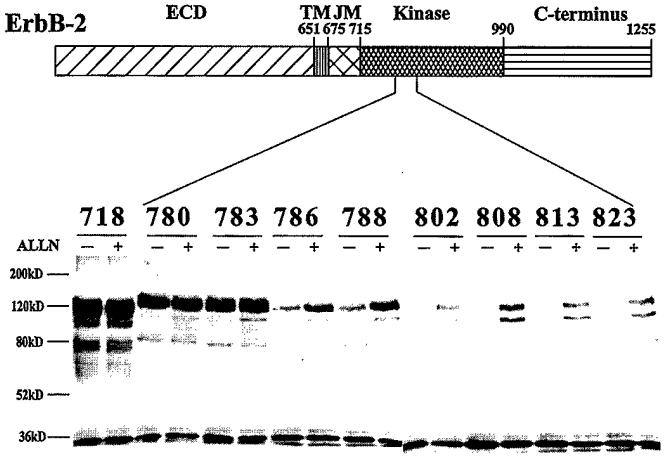

Fig. 5. Influence of kinase domain truncation mutations on ErbB-2 metabolic stability. Constructs encoding ErbB-2 mutants truncated at the indicated residues were expressed in Cos7 cells. Approximately 48 h after transfection, the cells were incubated with or without ALLN (250 μM) for 6 h. Equal aliquots of cell lysates were subjected to SDS-PAGE and Western blotting with antibody against ErbB-2 ectodomain.

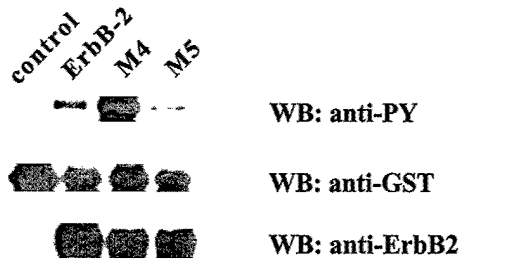

Fig. 6. Tyrosine kinase activity *in vitro* of ErbB-2 mutants. The ErbB-2 and the M3, M4, and M5 mutants were separately transiently expressed in Cos7 cells. Wild-type ErbB-2 or each mutant was precipitated with antibody against the ErbB-2 ectodomain. A kinase assay was then performed, using GST/PLC-γ1 SH2-SH2-SH3 domains as a substrate, as described in "Materials and Methods." The substrate was precipitated with GST antibody, electrophoresed, and blotted with phosphotyrosine or GST antibody. The level of ErbB-2 or mutant proteins present in the assay was detected by Western blotting with ErbB-2 antibody.

expression of these mutants was then assayed by transient expression in Cos7 cells. In each case, ALLN was added as indicated to block basal degradation of ErbB-2 mutants.

The results of this analysis, presented in Fig. 5, show when ErbB-2 is truncated within the kinase domain at residues 718, 780, or 783, the mutant proteins are expressed at a high level that is not affected by the presence of ALLN. However, when the truncation occurs at residue 786 or beyond, the mutant proteins are expressed at much lower levels, and their level of expression can be significantly increased by the presence of ALLN, suggesting that the low expression is caused by protein degradation. This result implies that sequence information between residues 784 and 786 increases the sensitivity of these ErbB-2 mutants to metabolic degradation.

To assess the significance of this sequence information, alanine mutagenesis was used to change the native ErbB-2 sequence LLGI to AAGA within the context of the full-length ErbB-2 molecule (mutant M5) and to test the potential role of this sequence in GA-induced degradation. It should be noted that this sequence is adjacent to the sequence (residues 776–783) shown previously in Fig. 4 (*i.e.,* mutant M4) to be necessary for GA-induced degradation of ErbB-2. In addition, this sequence is identified in the EGFR. The results, shown in Fig. 4, demonstrate that the M5 mutant is not sensitive to GA-induced degradation. Therefore, we have identified by somewhat distinct approaches two adjacent regions in ErbB-2, represented by mutants M4 and M5, that mediate GA-dependent degradation.

It might be argued that because each of these two mutations involve multiple substitutions, they may simply interfere with proper folding of the ErbB-2 kinase domain and thereby alter the receptor's sensitivity to GA. To assess this possibility, we have tested the tyrosine kinase activity of wild-type ErbB-2 and the M4 or M5 mutants using an *in vitro* kinase assay. The substrate in this kinase assay was a GST fusion protein that contains the tyrosine phosphorylation region of PLC-γ1 (Ref. 30).[4] The data in Fig. 6 show that both mutants phosphorylate this substrate and that the M4 mutant actually phosphorylates this substrate significantly more than wild-type ErbB-2. This assay shows that the kinase activity of these mutants is not grossly altered by these mutations. Hence, the folding of the kinase domains do not seem to be significantly altered by the M4 or M5 mutations.

As reported previously (27) and shown in Fig. 1, staurosporine and curcumin also stimulate ErbB-2 degradation in a manner that resembles that of GA in some aspects. Therefore, we tested whether the M4 and M5 mutants are resistant to degradation induced by staurosporine or curcumin as well as GA. The results, shown in Fig. 7, demonstrate that although each of the two ErbB-2 mutants are resistant to GA-induced degradation, each remains sensitive to staurosporine- or curcumin-induced degradation.

DISCUSSION

The data in this study indicate that after the addition of GA to cells, ErbB-2 is fragmented by a cleavage close to or within the tyrosine kinase domain. This is consistent with data published previously that have concluded that the kinase domain is necessary (18, 25, 27) and sufficient (27) for GA-induced degradation of ErbB-2. Cleavage within the kinase domain gives rise to fragments containing the NH_2 and COOH termini of ErbB-2. The NH_2-terminal fragment of 135 kDa is likely to include the ecto- and transmembrane domains plus a small part of the cytoplasmic domain. It is not clear, however, to what extent kinase domain residues are present in this fragment, because the sequence of its COOH terminus is not known.

As shown in this manuscript, several ErbB-2 fragments of similar but distinct molecular masses (42–51 kDa) are detected after the addition of GA. It is not clear whether each of these fragments is derived from a separate cleavage event within the ErbB-2 kinase domain or whether the cleavage of ErbB-2 produces one fragment that is then subject to the proteolytic processing. If multiple cleavages at distinct sites within the kinase domain did occur, this would be expected to result in molecular heterogeneity of both the NH_2- and COOH-terminal fragments. However, the 135 kDa NH_2-terminal fragment does not appear to be heterogeneous. Therefore, it seems more likely that post-ErbB-2 cleavage proteolytic processing of a single COOH-terminal fragment occurs to generate the multiple fragments that we have observed.

Using two different approaches, we have identified two adjacent sequence motifs that are located within the kinase domain of ErbB-2. When mutated, each of these motifs, represented by the mutations M4 and M5, produces a form of ErbB-2 that is not degraded in the presence of GA. Structural analyses of protein kinases indicate that each kinase domain is composed of an NH_2- and COOH-terminal lobe. The GA-resistant mutations that we have identified lie within the NH_2-terminal lobe whose major function is to facilitate the binding of ATP for subsequent catalysis. Sequence homology analysis has divided kinase domains into ~11 distinct subdomains that can be aligned with structural characteristics, such as β sheets and α helices. The M4 and M5 mutants encompass 13 residues that begin near the end of subdomain III and extend through subdomain IV. This places the M4 residues in a connecting strand between the αC helix and β4 strand, whereas the M5 residues are within the β4 strand. This part of the NH_2-terminal lobe is not directly involved in kinase catalytic

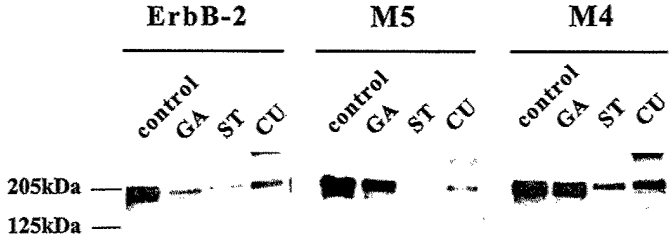

Fig. 7. Selectivity of ErbB-2 mutants to drug-induced metabolic degradation. Wild-type ErbB-2 and the M4 and M5 mutants were separately expressed in Cos7 cells. Approximately 48 h later, the cells were incubated for 6 h with GA (3 μM), staurosporine (*ST*; 5 μM), or curcumin (*CU*; 40 μM). The cells were lysed, and equal aliquots of protein were electrophoresed and blotted with antibody against the ErbB-2 extracellular domain.

[4] D. Tvorogov and G. Carpenter, unpublished data.

function. Hence, it seems understandable that the M4 and M5 mutations do not abrogate kinase activity.

Although the M4 mutation changes a unique sequence in ErbB-2 to resemble the corresponding sequence in the EGFR, which is not sensitive to GA, the M5 mutation changes an ErbB-2 sequence that is preserved in the EGFR. Hence, although the sequence at residues 784–786 of ErbB-2 may be necessary for GA-induced degradation, it is clear that these residues are not sufficient to mediate sensitivity to this drug. It seems likely, given their proximity, that the ErbB-2 sequences defined by the M4 and M5 mutations cooperate to facilitate GA-induced degradation of this molecule.

The mechanism by which GA initiates ErbB-2 degradation has been proposed to involve the binding of Hsp90 to ErbB-2 (25). Hsp90 also binds GA, and in the presence of GA, Hsp90 association with ErbB-2 is disrupted. The mutations to GA resistance reported in this study could represent residues involved in Hsp90 binding to ErbB-2. If these mutations did abrogate Hsp90 binding, we would expect the mutants to become more sensitive to degradation in the absence of associated Hsp90. A second possibility is that the mutations affect protease recognition of ErbB-2 or alter the kinase domain cleavage site. The identity of the protease(s) that cleaves ErbB-2 within the kinase domain in the presence of GA is unknown. However, the kinase domain M4 and M5 mutations prevent GA-induced cleavage but do not block cleavage induced by staurosporine or curcumin, which produces similar sized fragments from ErbB-2. Hence, this model would require that GA-induced degradation of ErbB-2 involves protease distinct from those involved in staurosporine- or curcumin-induced ErbB-2 degradation.

Lastly, it is possible that cleavage of the ErbB-2 molecule produces fragments that may have biological effects in cells. This has been reported for other tyrosine kinases, such as RET, that are subject to cleavage within their cytoplasmic domain by caspases (31). This possibility is being evaluated.

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Identification of proteolytic fragments from ErbB-2 that induce apoptosis

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Running title: pro-apoptotic peptides from ErbB-2 kinase domain

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ABSTRACT

The receptor tyrosine kinase ErbB-2 plays an important role in cell proliferation and differentiation as well as oncogenesis. We have found that ErbB-2 kinase domain fragmentation is important for induction of apoptosis. Exogenous expression of peptides derived from the ErbB-2 kinase domain induce cells death with the hallmarks of apoptosis. In contrast, transfection of the ErbB-2 carboxyterminal domain did not induce apoptosis. We have identified a 38-residue segment from the ErbB-2 kinase N-terminal lobe that can strongly induce apoptosis in transfected cells. Cell death was not blocked by the pan-caspase inhibitor z-VAD-FMK. Similar fragments derived from several other receptor tyrosine kinases also induce cell death. These data imply that proteolytic fragmentation of tyrosine kinases liberate apoptotic fragments that can accelerate cell death.

ErbB-2 is an orphan receptor that functions through the formation of heterodimers with all other members of the EGF receptor family. Overexpression of ErbB-2 has been demonstrated in 25-30% of human breast and other cancers and correlates with an aggressive form of the disease and a poor prognosis (Yarden Y. and Sliwkowski M., 2001). Signaling through ErbB-2 elicits a strong proliferative response in many cell lines and plays an important role in regulating cell growth and differentiation. In addition, the ErbB-2 receptor is necessary for the invasion of carcinoma cells (Neve R. *et al.*, 2001).

The susceptability of ErbB-2 to extracellular manipulations and its increased level of expression in tumors means that it is an attractive target for therapeutic interventions, which currently include pharmacological drugs that reduce ErbB-2 levels in cancer cells and antibodies against the ErbB-2 extracellular domain, whose mechanism of action is not clear, but often includes increased degradation of ErbB-2.

Published data suggest that the presence of increased levels of ErbB-2 is important for tumor cells to exibit sensitivity to ansamycin antibiotics, such as geldanamycin, or antibodies against ErbB-2, including Herceptin, which is in use in clinical practice. Either geldanamycin or Herceptin induce an increased rate of apoptosis in tumor cells that express enhanced levels of ErbB-2, as demonstrated in experiments with isogenic tumor cells expressing low or high levels of ErbB-2 (Smith *et al.*, 2002, Munster *et al.*,2002, Cuello M. *et al*, 2001). It has been shown that geldanamycin provokes increased ErbB-2 degradation by binding to the chaperone Hsp90 and inhibiting its association with ErbB-2 (Xu W. *et al*, 2001). However, the

molecular mechanisms of geldanamycin- or Herceptin-induced apoptosis are poorly understood.

Previous studies have shown that the induction of ErbB-2 degradation in breast cancer cells by drugs, such as geldanamycin, curcumin or staurosporin, involves the formation of discrete receptor fragments (Tikhomirov and Carpenter, 2003). The addition of these drugs stimulate several cleavage events within the ErbB-2 cytoplasmic domain. For example, caspase-dependent cleavages are induced within the ErbB-2 carboxyterminal domain and generate several overlapping fragments of the ErbB-2 molecule, which can be detected with antibodies against the ErbB-2 carboxyterminal domain (Tikhomirov and Carpenter, 2001, 2003). Also, it has been demonstrated that treatment of ErbB-2 expressing cells with geldanamycin induces cleavage within its kinase domain, which generates a 135 kDa fragment (Tikhomirov and Carpenter, 2000). Transfection of such large ErbB-2 fragments, which contain the extracellular domain, the transmembrane domain and part of the kinase domain, results in the rapid degradation of these fragments if they include the RLLGI motif (residues 783-788) present in the ErbB-2 kinase domain. In contrast, transfected ErbB-2 fragments created by truncation upstream of this motif, are metabolically stable (Tikhomirov and Carpenter, 2003).

To determine the biological activity of such ErbB-2 fragments, constructs that contain the ErbB-2 extracellular domain, transmembrane domain and portions of the kinase domain truncated at either residues 829 (Δ829) or 927 (Δ927), as depicted in Figure 1A, were generated. Each construct was fused at the C-terminus to GFP. As a control, ErbB-2 truncated close to its carboxyterminus at residue 1216 (Δ1216) and fused to GFP was used.

Figure 1

As reported previously, ErbB-2 fragments truncated within the kinase domain were metabolically unstable after transfection into Cos7 cells and their expression levels were much lower than that for ErbB-2 Δ1216 (Figure 1A, right panel). When tested for cell growth, almost all transfected cells expressing ErbB-2 Δ829 or Δ927 died in 3-4 days (Figure 1B). In contrast, cells expressing ErbB-2 Δ1216 survived for at least 14 days and eventually formed cell clusters (Figure 1C, left panel). Expression of ErbB-2 constructs Δ829 or Δ927 is associated with dramatic morphological and biochemical changes consistent with apoptosis (Figure 1C, right panel). These changes include a rounded cell shape with remarkably reduced cytoplasm, shrinkage of the nucleus, chromatin condensation, detachment from substrate, and eventually cell death. Therefore, after 3-4 days following transfection only a few cells expressing ErbB-2 truncated proteins Δ829 or Δ927 could be detected, which is strikingly different from the control (Δ1216) cells. It should be noted that while all three constructs contain the RLLGI motif, only the Δ829 and Δ927 constructs resemble cleavage that occurs within the ErbB-2 kinase domain downstream of this motif.

To exclude the possible influence of GFP in these results, Cos7 cells were also transfected with a construct similar to ErbB-2 Δ829, which encodes an approximately 135 kDa ErbB-2 fragment containing residues 1-802 (Δ802) but does not contain GFP, or wild-type ErbB-2 as a control. The cells were stained with an antibody against ErbB-2 ectodomain. Transfection of Cos7 cells with the Δ802 ErbB-2 fragment, but not with a wild-type ErbB-2, resulted in cell death as shown by the data in Figure 1D.

Lastly, GFP-fused constructs encoding ErbB-2 truncated within the kinase domain or GFP alone were transfected into CHO, 3T3, A431, MCF7 and SKBr3 cells.

In each case the ErbB-2 truncation construct, but not GFP, induced cell death (data not shown). Therefore, these data indicate that ErbB-2 fragments created by a truncation within the kinase domain produce a cell death response in multiple cell lines.

To further define the ErbB-2 fragments that induce cell death, Cos7 cells were transfected with a vector encoding residues 679-802 in the N-terminal lobe of the ErbB-2 kinase domain fused to GFP (Figure 2A) or GFP as a control. Transfected and untransfected cells were separated using flow cytometry. Cell populations expressing the ErbB-2 peptide 679-802 or GFP alone were plated and grown for 2 days in Titertek chambers. The results clearly show that cells transfected with GFP alone spread on the growing surface and survived for at least seven days (Figure 2B, left panel). In contrast, all cells transfected with the ErbB-2 residues 679-802 fused to GFP exibit apoptotic morphologic changes (Figure 2B, right panel) and die in 2-3 days (Figure 2C). The level of expression of this peptide in individual Cos7 cells was different and cells with the highest levels of expression died on the first or second day after transfection. However, even cells that express low levels of this GFP fusion protein die 3-4 days after transfection showing a strong cytotoxic effect of this ErbB-2 fragment. **Figure 2**

To define a minimal ErbB-2 peptide able to induce cell death, four additional constructs encoding different segments from the ErbB-2 kinase domain fused to GFP were prepared (Figure 2A). Expression in Cos7 cells of the ErbB-2 peptide 679-783, which does not include the RLLGI motif (residues 783-788), did not stimulate apoptosis. However, expression of ErbB-2 peptides that encompass residues 765-813 and 765-802, which do contain the RLLGI motif, were sufficient to induce cell death in transfected cells. Also, a shorter peptide that contains residues 765-783 was ineffective in induction of cell death after expression in Cos7 cells. Therefore, we

concluded that peptides derived from the ErbB-2 kinase domain that include the RLLGI motif and surrounding residues stimulate cell death in transfected cells.

Since the RLLGI motif is conserved among receptor tyrosine kinases, we expressed, as GFP fusion proteins, the corresponding residues of the N-terminal lobe of several other tyrosine kinases, i.e. ErbB-1 (residues 651-803), ErbB-4 (residues 744-823), TrkA (residues 540-632), VEGFR1 (residues 821-927). As shown in Figure 3, when these constructs are transfected in Cos7 cells, each strongly induced apoptosis. In contrast, constructs representing the carboxyterminal domains of ErbB-1 (residues 960-1186) or ErbB-2 (residues 1140-1220) fused to GFP were inefficient in the induction of cell death (Figure 3B and data not shown). **Figure 3**

In order to determine whether caspase activation is necessary for induction of apoptosis by ErbB-2 fragments, Cos7 cells, transfected with GFP fused to ErbB-2 residues 679-802, were treated with the pan-caspase inhibitor z-VAD-FMK (10-50 μM) or inhibitors of individual caspases. None of these inhibitors blocked induction of cell death (data not shown).

Our observation that peptides derived from the tyrosine kinase domain stimulate cell death suggests that proteolytic fragmentation of ErbB-2 may be at least partially responsible for apoptosis observed in the presence of several drugs that stimulate cleavage of the tyrosine kinase domain. It is important to note that such fragments generated in response to drug treatment are quickly degraded in the cytosol (Tikhomirov and Carpenter, 2003) and, therefore, are unlikely to activate an apoptotic program in cells expressing low levels of ErbB-2. However, if there are elevated levels of ErbB-2 in a cell, the amount of such fragments produced in response to compounds, such as geldanamycin, is also increased, which may stimulate or at least contribute to apoptosis observed in these cells.

It has been reported that overexpression of many serine/threonine protein kinases induces a strong apoptotic response after caspase-mediated cleavage. This growing list includes PKCδ (Datta R. *et al.*, 1996), PKCμ (Endo K. *et al.*, 2000), DAP1 (Inbal B. *et al.*, 2000), PAK-2 (Rudel T. and Bokoch G., 1997), Dlk/ZIP (Kogel D. *et al.*, 1999), Etk/Bmx (Wu Y. *et al.*, 2001), PKN (Takahashi M. *et al.*, 1998), ROCK1 (Sebbagh M. *et al.*, 2001), HPK1 (Chen Y. *et al.*, 1999), SLK (Sabourin L. et al., 2000), RIP1 (Kim J. et al., 2000), RIP3 (Sun X. et al., 1999), MST1 (Ura S. *et al.*, 2001), and others. It has been proposed recently that proteolytic activation of protein kinases could be a general mechanism of apoptotic signal transduction (Bokoch G, 1998). In many published reports, cleavage of protein kinases by caspases is followed by an increase in kinase activity, which may result in activation of JNK and other downstream protein kinases involved in apoptotic responses.

However, other reports show that kinase activation is not necessarily involved in the induction of apoptosis following protein kinase cleavage mediated by caspases. Overexpression of the RET tyrosine kinase strongly induces apoptosis, which is dependent on caspase activation and RET cleavage, but does not require tyrosine kinase activation (Bordeaux M *et al.*, 2000). It has been suggested that MEKK1 and MEKK4 cleavage or expression of the truncated protein kinases, which contain the kinase domain, can generate proteolytically processed pro-apoptotic fragments (Bonvin C. *et al.*, 2002). Cleavage of death domain kinase RIP by caspase 8 without kinase activation is essential for TRAIL-induced apoptosis (Lin Y. *et al.*, 2000). Ask1 protein kinase lacking the kinase activity is efficient as wild type Ask1 in induction of apoptosis (Charette S. *et al.*, 2001). These results suggest that caspase-dependent cleavage of protein kinases and the generation of pro-apoptotic fragments

may be a general fenomenon. Since there is a large number of proteins kinases in a cell, this implies that during induction of apoptosis multiple cleavages within their kinase domains may release proteolytic fragments, which will further accelerate the progress of apoptosis. This could represent a new mechanism contributing to the induction of apoptosis.

Acknowledgments: The support of the Department of Defense Idea Award Grant DAMD 17-00-1-0483, grant of the Vanderbilt Ingram Cancer Center P30 CA68485 and the Vanderbilt Diabetes Center P30 DK20593 for core facility support are acknowledged.

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FIGURE LEGENDS

Figure 1. **Transfection of Cos7 cells with truncated ErbB-2 receptor induces cell death**. The ErbB-2 receptor fused C-terminally to GFP was prepared using primers CCCATCTGCACCATCGATGTCCAC (5') and TCTAGACTCGTCGACTGGCACG TCCAGACCCAGGTA(3') to amplify C-terminal part of the ErbB-2. GFP cDNA was amplified in PCR using primers CTACCGGTCGTCGACATGGTGAGCAAGGGCGAG GAG (5') and AGATCTTCTAGACTACTTGTACAGCTCGTCCATGCC. Each primer was cut with Sal I and both primers were ligated. The product of ligation was cut with Kpn I and Xba I and inserted into pcDNA/ErbB-2 vector cut with Kpn I and Xba I. Primers GTAATAGAG GTTGTCGACGGCTGG, GTAGCTCATCCCGTCGACAAT CTG, or GTCAGGGAT CTCTGCGACTGGGAT were used to prepare ErbB-2 constructs truncated at residues 1216, 829, 927, respectively. The cells were grown in DMEM containing 10 % FBS and transfected with ErbB-2 inserted into pcDNA3.1+ vector and truncated at residues 1216 (Δ1216), 927 (Δ927), or 829 (Δ927) (A, left panel). The expression of corresponding proteins was confirmed by western blot (A, right panel). Next, the cells were grown for ten days (Δ1216, Figure 2C, left panel) or four days (Δ829, Figure 2C, right panel) and images were prepared using fluorescent microscopy. The difference in number of transfected cells between the ErbB-2 Δ1216 and ErbB-2 Δ829 is shown quantitatively in panel B.

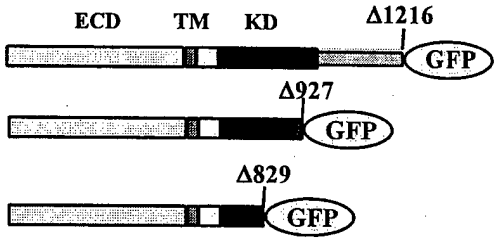
D. Cos 7 cells were transfected with ErbB-2 (left) or ErbB-2 peptide truncated within the kinase domain at residue 802 (Δ802) that is not fused to GFP (panel D, right). The cells were fixed in 4% paraformaldehyde and stained using antibody against ErbB-2 extracellular domain (Calbiochem, c-neu antibody 5).

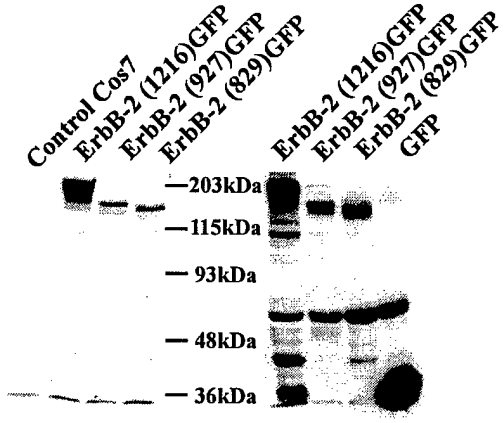
Figure 2. **Determination of a minimal peptide derived from the ErbB-2 kinase domain that induce cell death upon transfection.** GFP fusion proteins encoding peptides derived from the ErbB-2 kinase domain were prepared by PCR using primers CTCATCAAGCGAGCTCAGCAGAAGATC (5'), AACACATCCCCCAGAGCTCAC AGAGAAATCTTA (5'), GTCTAAGAGGTCGACCTAGGGCATAAGCTGTGTCAC CAG (3'), GGAGCCCAGGTCGACTCAGTTTTCCCGGACATGGTCTAA (3') CAC CGTGGAGTCGACTCAGATGCCCAGAAGGCGGGAGAC (3') and inserted into pEGFP-C1 vector through Sac I and Sal I restriction sites (Panel A). The cells were grown for 2 days and then were sorted using flow cytometry to separate transfected from untransfected cells. Sorted cells were then plated again and grown for 2 days. Transfection of peptides encompassing residues 679-802, 765-813, and 765-802, which include RLLGI morif, results in cell death of transfected cells (Panel B, right). In contrast peptides 679-783, or 765-783 derived from the ErbB-2 kinase domain, which do not contain this motif, or ErbB-2 C-terminus (residues 1140-1220) fused to GFP were not able to induce cell death (Panel B, left). The cells were incubated for indicated times after transfection. The number of transfected cells expressing individual GFP fusion proteins is shown in panel C.

Figure 3. **Cell death induced by expression of peptides derived from the kinase domain of EGFR, VEGFR1, ErbB-4, or TrkA.** Peptides including N-terminal part of the kinase domain corresponding to ErbB-2 apoptotic segment 679-802 were prepared by PCR with primers EGFR: CGAAGGCGCCACAGAGCTCGGAAGCGCACG (5'), CAAGCGGTCGAC

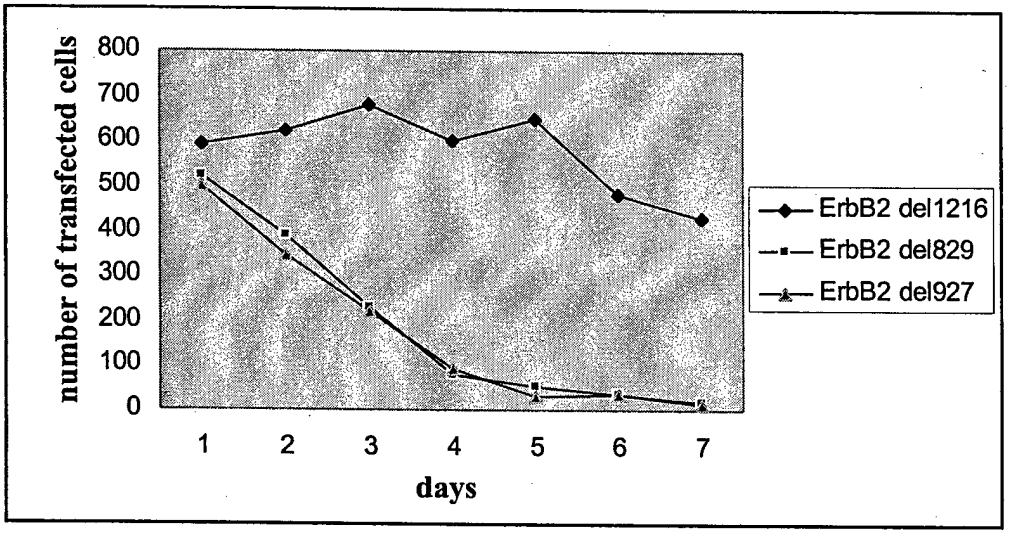
TCAGTAGTTCATGCCCTTTGCGAT (3'); VEGFR1: GATGCCAGCAGAGCTCAA TTTGCCCGGGAGAGACTTAAA (5'), ATCCTTGTTGTCGACCTATAAGTCACG TTTGCTCTTGAG (3'); ErbB-4: GTACCTGAAGGAGCTCCTGTGAAGATTCCT GTG (5'), CTTAGCTCTGTCGACTCACCAGTTAAGCAGCAGTTG (3'); TrkA: GAG CAGGACAGAGCTCAAGTGGCTGTCAAGGCACTGAAGGAG (5'), CATCCCCGC GTCGACCTAGCTAGCCACGGCCAGCAGCTG CC (3') and inserted into pEGFP-C1 vector through Sac I and Sal I restriction sites. Expression of all peptides derived from the kinase domain of the corresponding receptor tyrosine kinases results in massive cell death. In contrast, C-terminal parts of ErbB-2 or EGF receptor as well as GFP did not induce cell death (Panel A). The cells were incubated for indicated times and cells expressing GFP fusion proteins were counted using fluorescent microscopy (Panel B).

A

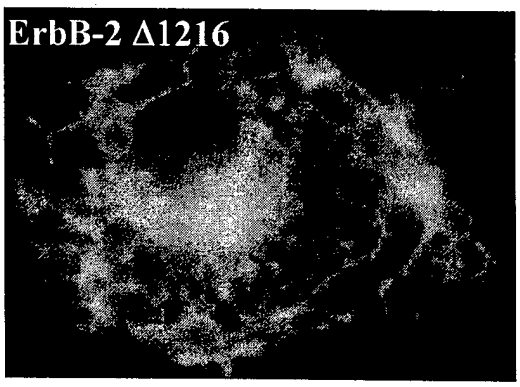


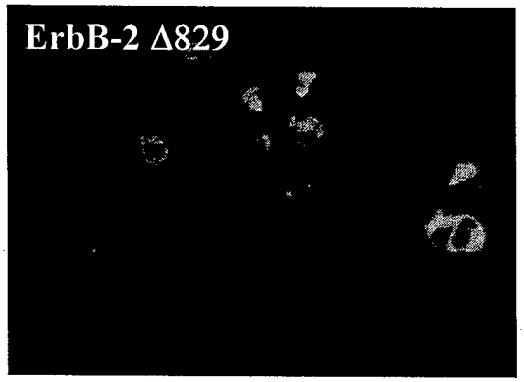


B



C





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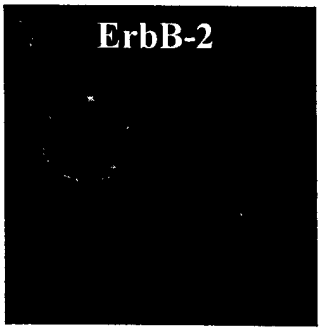


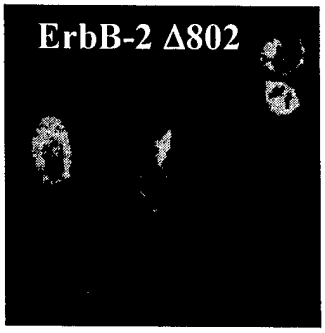
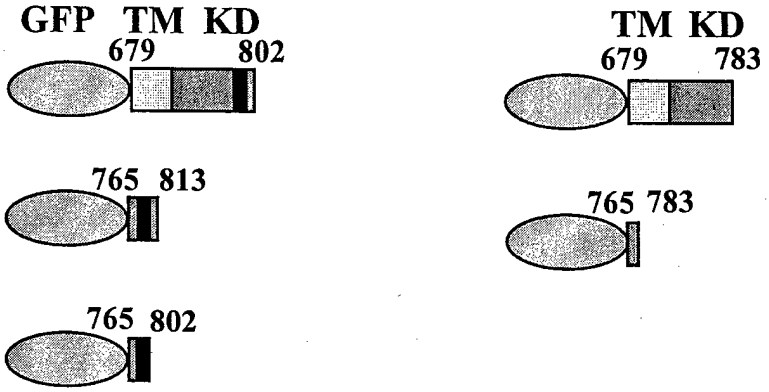


Figure 1

A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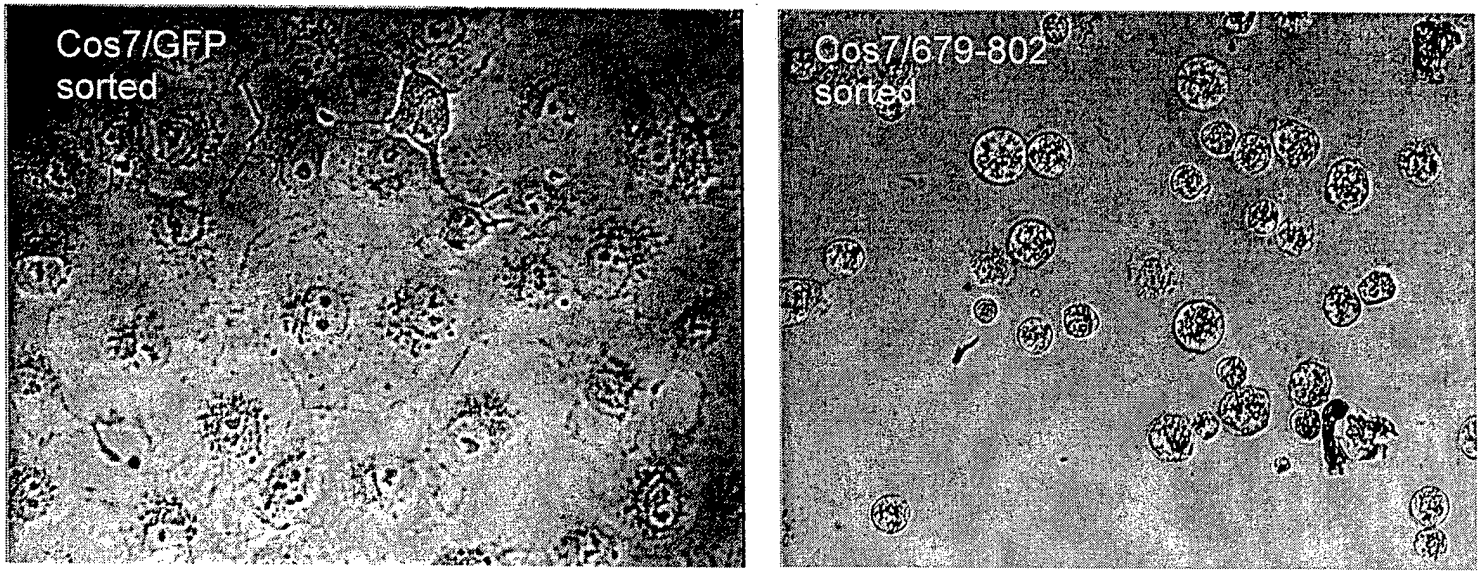
679
802
765 813
765 802
TM KD
679
783
765 783

B


Cos7/GFP
sorted
Cos7/679-802
sorted

C

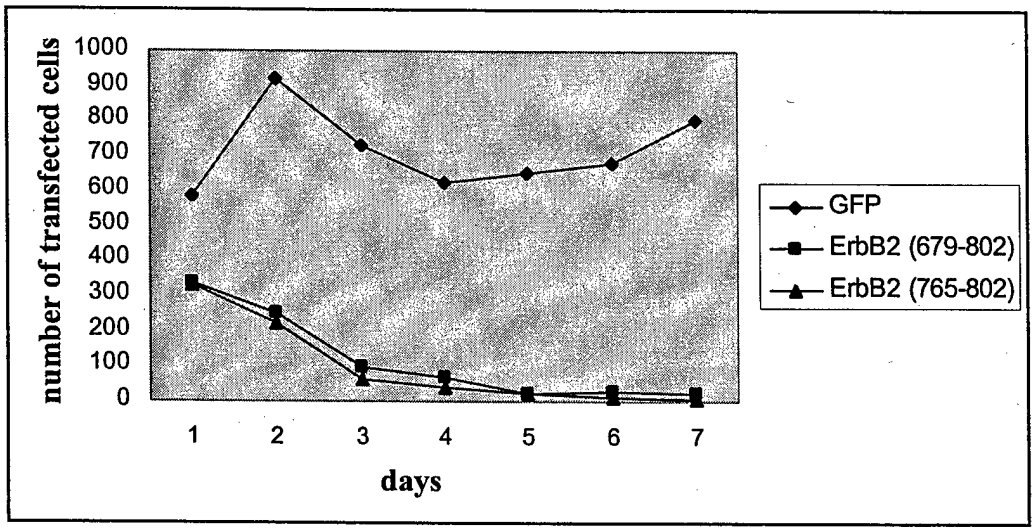
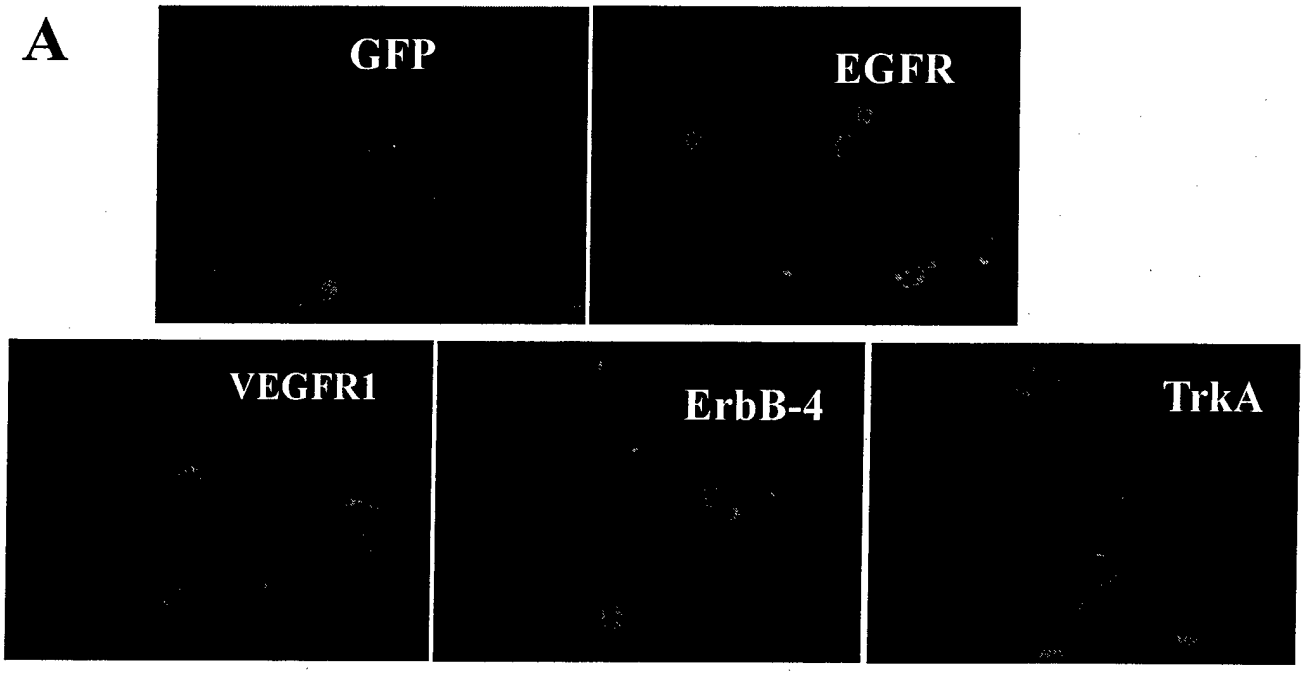

number of transfected cells
1000
900
800
700
600
500
400
300
200
100
0
1
2
3
4
5
6
7
days
GFP
ErbB2 (679-802)
ErbB2 (765-802)

Figure 2


A
GFP
EGFR
VEGFR1
ErbB-4
TrkA

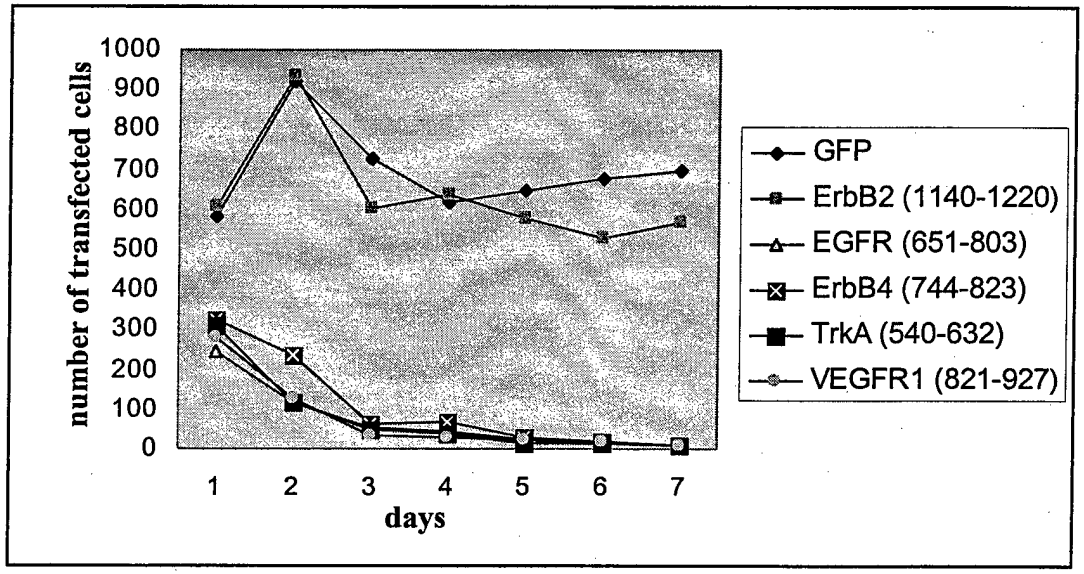

B
number of transfected cells
1000
900
800
700
600
500
400
300
200
100
0
1
2
3
4
5
6
7
days
GFP
ErbB2 (1140-1220)
EGFR (651-803)
ErbB4 (744-823)
TrkA (540-632)
VEGFR1 (821-927)

Figure 3